“Discovery consists in seeing what everyone else has seen and thinking what no one else has thought.”
- Albert Szent-Gyorgyi

“Live through this and you won’t look back.”
-Stars

University of Alberta

Structural and Functional Analysis of the Type IV Pili from *Pseudomonas aeruginosa*

by

Erin van Schaik ©

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Abstract

Pseudomonas aeruginosa is an opportunistic pathogen that is a major cause of morbidity and mortality in infected individuals. Major virulence determinants of *P. aeruginosa* are type IV pili, which are polar, filamentous, surface appendages. The ability to bind and move across surfaces through pilus-mediated twitching motility facilitates biofilm formation, which is also dependent on exogenous DNA. Biofilms can form on abiotic surfaces and biotic surfaces such as the lungs of cystic fibrosis patients.

It was determined that the type IV pili from *P. aeruginosa* are able to bind directly to DNA. Pilus-mediated DNA binding is dependent on the intact pilus structure and involves backbone interactions with preferential binding to pyrimidine residues even though there is no evidence of sequence specific binding. Pilus-mediated DNA binding has important implications for biofilm formation as DNA increases *P. aeruginosa* colonization to stainless steel and decreases colonization of epithelial cells.

Pilus-mediated DNA binding is inhibited by a monoclonal antibody (MAb) PKL1. Even though MAb PKL1 recognizes a 3-dimensional epitope that is not present in the C-terminal receptor binding domain (RBD), MAb PKL1 can inhibit cellular binding by 80%, which has implications for vaccine designs. Characterization of MAb PKL1 suggested that there are multiple conformations of the C-terminal RBD in the intact pilus, which may effect binding mediated functions. In addition, it was determined that the C-terminal RBD is exposed

along the length of the pilus, which supports a 3-start left-handed helix model for the intact *P. aeruginosa* type IV pili.

Quorum sensing autoinducers are released from *P. aeruginosa* and are candidates to mediate changes in the binding affinity of the type IV pilus. In addition to altering genetic control in *P. aeruginosa* the homoserine lactone autoinducers also modulate type IV pilus binding functions. Moreover, the modulation by autoinducers is partially dependent on functional twitching motility ATPases. I propose that the quorum sensing autoinducers modulate pilus mediated binding through an allosteric mechanism.

The structural and biochemical analysis of the type IV pili from *P. aeruginosa* has therefore identified new ligands and a novel mechanism to control pilus-mediated binding functions.

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For my mother, Allie
who taught me to believe in myself

Table of Contents

1. General Introduction....2
    - 1.1. *Pseudomonas aeruginosa*....2
    - 1.2. *P. aeruginosa* infections....4
    - 1.3. *P. aeruginosa* chronic lung infections in cystic fibrosis patients....5
    - 1.4. *P. aeruginosa* virulence factors....7
    - 1.5. Global regulation of virulence....11
    - 1.6. Type IV pili....17
    - 1.7. Structures of type IV pilins....22
    - 1.8. Assembly of type IV pili....31
    - 1.9. Functions of type IV pili....37
        - 1.9.1. Colonization....39
        - 1.9.2. Natural transformation....41
        - 1.9.3. Twitching motility....42
        - 1.9.4. Biofilm formation....44
    - 1.10. Objectives....49

2. DNA Binding: A novel Function of *P. aeruginosa* Type IV Pili....51
    - 2.1. Introduction....52
    - 2.2. Material and methods....54
        - 2.2.1. Bacterial strains, DNA source, and antibodies....54
        - 2.2.2. Pili purification, biotinylation, and confirmation of activity....56
        - 2.2.3. Direct DNA binding assays....58
        - 2.2.4. Competitive DNA binding assays....59
        - 2.2.5. Direct and competitive oligonucleotide binding assays....59
        - 2.2.6. Antibody inhibition assays....60
    - 2.3. Results....61
        - 2.3.1. Pilin electrostatics and structural based hypothesis for a pilus-associated DNA binding function....61
        - 2.3.2. Pilus mediated DNA binding....62
        - 2.3.3. Pilus mediated DNA binding is a specific event that involves interactions with the DNA backbone....66
        - 2.3.4. Pilus mediated DNA binding is not sequence specific although binding to pyrimidine bases is preferred....71
        - 2.3.5. DNA binding is a tip-associated event that is dependent on the quaternary structure of the pilus....75
    - 2.4. Discussion....77

3. Multiple Epitopes are Associated with the C-Terminal Receptor Binding Domain of *Pseudomonas aeruginosa* Type IV Pilus....86
    - 3.1. Introduction....87
    - 3.2. Material and methods....91
        - 3.2.1. Bacterial strains....91

3.2.2. Peptide synthesis and conjugation to protein carriers........92
3.2.3. Enzyme linked immunoassays........93
3.2.4. Transmission immuno-electron microscopy........95
3.2.5. Direct binding assays........96
3.3. Results........97
3.3.1. Antibody titers to PAK pili and PAK pilin........97
3.3.2. Antibody titers to PAK pilin peptides........100
3.3.3. PKL1 Inhibits PK99H epitope recognition........102
3.3.4. PKL1 binds close to or at the pilus tip and inhibits binding.....103
3.3.5. The C-terminal RBD is exposed at the tip and along the length of the PAK type IV pilus........108
3.3.6. Antibodies specific for the C-terminal RBD can detect PAK pili bound to cellular receptors........109
3.4. Discussion........113
3.4.1. PKL1 epitope specificity........116
3.4.2. Implications for the structure and binding functions of the PAK type IV pilus........115

4. Quorum Sensing Autoinducers are Allosteric Regulators of *Pseudomonas aeruginosa* Type IV Pili, Which has Implications for Biofilm Formation........121
4.1. Introduction........122
4.2. Material and methods........125
4.2.1. Bacterial strains, DNA, and PAIs........125
4.2.2. Biotinylation of *P. aeruginosa* cells and purified pili........126
4.2.3. Stainless steel binding assays........126
4.2.4. Cellular binding assays........128
4.3. Results........129
4.3.1. *P. aeruginosa* autoinducers modulate cellular binding mediated by type IV pili........129
4.3.2. *P. aeruginosa* autoinducers modulate the binding of type IV pili to a variety of acellular substrates........132
4.3.3. The *P. aeruginosa* autoinducers do not modulate pilus-mediated binding of hyperpiliated twitching motility mutants....136
4.3.4. DNA affects the initial colonization of stainless steel and buccal epithelial cells mediated by *P. aeruginosa* type IV pili........141
4.4. Discussion........147

5. General Discussion........157
5.1. Pilus-mediated DNA binding and biofilm formation........158
5.2. Cis/trans isomerization of a conserved proline may mediate the allosteric regulation of the *P. aeruginsoa* type IV pili........160
5.3. Future directions........164

6. Bibliography..167

Curriculum Vitae..224

List of Tables

Table 1-1. Proteins required for type IV pilus biogenesis in several gram-negative bacterial species....................34

Table 2-1. Oligonucleotides used for direct and competitive inhibition assays...72

Table 3-1. The synthetic PAK pilin peptides of the C-terminal RBD used either un-conjugated or conjugated to KLH or BSA....................94

List of Figures

Figure 1-1. Transmission electron micrograph of *P. aeruginosa* strain K............3
Figure 1-2. The structure of the quorum sensing autoinducers expressed by *P. aeruginosa*..13
Figure 1-3. A simplified schematic diagram representing the global control of the quorum sensing regulon..15
Figure 1-4. A simplified schematic diagram depicting the switch between acute and chronic infection..18
Figure 1-5. The structures of the type IVa N-terminal truncated pilins..............23
Figure 1-6. The structures of the type IVb N-terminal truncated pilins..............24
Figure 1-7. The 3-start right-handed hypothetical PAK pilus model with four subunits per turn proposed by Craig *et al.* (2004)................................30
Figure 1-8. A simplified schematic of pilus assembly based on the results presented by Nunn and Lory (1991), Crowther and colleagues (2004), and Wolfgang *et al.* (2000)..38
Figure 1-9. A schematic diagram of the separate stages that occur during biofilm formation...46

Figure 2-1. Amino acid sequence and structural aspects of *P. aeruginosa* type IV pilins...55
Figure 2-2. Purification of type IV pili from *P. aeruginosa* DB2 expressing KB7 pili, DB2 expressing K122-4 pili, and PAK2Pfs.....................................57
Figure 2-3. Direct binding of purified biotinylated PAK pili to asialo-GM$_1$...........64
Figure 2-4. Concentration dependence of DNA binding by purified biotinylated pili from *P. aeruginosa* strains PAK, K122-4, and KB7..................65
Figure 2-5. Exogenous DNA inhibits binding or biotinylated pili to immobilized DNA. pUCP19 plasmid..68
Figure 2-6. Unlabeled PAK pili inhibit binding of biotinylated PAK pili to immobilized DNA...69
Figure 2-7. Polyphosphate and not pyrophosphate inhibits binding of biotinylated pili to immobilized DNA..70
Figure 2-8. Direct and competitive binding assays using biotinylated oligonuceotides to immobilized *P. aeruginosa* pili purified from strains PAK, K122-4, and KB7..74
Figure 2-9. Antibody inhibition of binding biotinylated PAK pili to immobilized pUCP19...76
Figure 2-10. The synthetic peptide PAK(128-144)ox representing the C-terminal receptor binding domain does not inhibit binding of biotinylated PAK pili to immobilized DNA..78

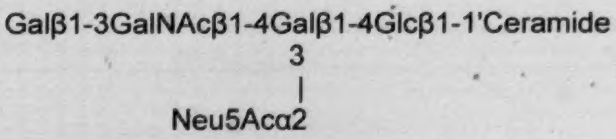
Figure 3-1. Whole cell ELISA using *P. aeruginosa* strains PAK a wild type strain, PAKΔPil a pilus-deficient strain, and PAK2Pfs a hyperpiliated strain......99
Figure 3-2. Direct ELISA using PAK pilin peptides from the C-terminal

receptor binding domain conjugated to BSA..101
Figure 3-3. Indirect competitive ELISA assay using PKL1 and PK99H...........104
Figure 3-4. Inhibition of pilus-mediated cellular binding by monoclonal antibodies PKL1 and PK99H, or PAK(128-144)ox peptide..........................105
Figure 3-5. Inhibition of pilus-mediated DNA binding by monoclonal antibodies PKL1 and PK99H..107
Figure 3-6. The C-terminal RBD is exposed along the length of the PAK pilus not just at the tip..110
Figure 3-7. Detection of PAK pili bound to cellular receptors using monoclonal antibodies specific for the C-terminal RBD..............................112
Figure 3-8. Structure of PAK monomeric pilin (Δ1-28) (Hazes *et al.*, 2000)......115
Figure 3-9. The 3-start left-handed helix model proposed by Craig *et al.* (2004)..119

Figure 4-1. Exogenous synthetic PAIs increase pilus-mediated binding to BECs and asialo-GM_1 in a concentration dependent manner....................131
Figure 4-2. The effects of exogenous synthetic PAIs on the colonization of *P. aeruginosa* strains PAK and PAK NP to stainless steel.......................133
Figure 4-3. Exogenous synthetic PAIs increase the binding of *P. aeruginosa* PAK cells and purified pili to stainless steel in a concentration dependent manner...134
Figure 4-4. The effects of an exogenous hydrophbe on the binding of *P. aeruginosa* PAK cells or purified PAK pili to stainless steel.........................135
Figure 4-5. Exogenous PAIs increase pilus-mediated binding to immobilized DNA in a concentration dependent manner...137
Figure 4-6. The effects of exogenous synthetic PAIs on the adherence of *P. aeruginosa* strains PAK R364 or PAK S34 to stainless steel.........................139
Figure 4-7. The effects of exogenous synthetic PAIs on *P. aeruginosa* PAK cells and isogenic mutants after colonization of stainless steel..............140
Figure 4-8. The effects of exogenous DNA on the colonization of *P. aeruginosa* strains PAK and PAK NP to stainless steel...........................142
Figure 4-9. Exogenous DNA increases the *P. aeruginosa* colonization of stainless steel...143
Figure 4-10. Exogenous DNA decreases the colonization of *P. aeruginosa* to BECs..146

List of Abbreviations

A	absorbance
ABTS	2,2'-Azino-di-[3-ethylbenzthiazoline-6-sulfonic acid diammonium salt
asialo-GM_1	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1'Ceramide
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
BCA	bicinchoninic acid
BEC	buccal epithelial cell
Bfp	bundle forming pili
BLAST	basic local alignment search tool
BSA	bovine serum albumin
bp	base pairs
cAMP	cyclic adenosine 5'-monophosphate
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance channel
CFU	colony forming unit
CRP	catabolite repressor protein
dH_2O	distilled water
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunoassay
EM	electron microscopy
EMSA	electrophoretic mobility shift assays
EPEC	enteropathogenic *Escherichia coli*

EPS	extracellular polymeric substance
GAP	GTPase activation domain
GM$_1$	 $\mathrm{Gal}\beta1\text{-}3\mathrm{GalNAc}\beta1\text{-}4\underset{\underset{\mathrm{Neu5Ac}\alpha2}{\overset{3}{\vert}}}{\mathrm{Gal}}\beta1\text{-}4\mathrm{Glc}\beta1\text{-}1'\mathrm{Ceramide}$
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IgA	immunoglobulin A
IgG	immunoglobulin G
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
kDa	kilodaltons
KLH	keyhole limpet hemocyanin
L-B	Luria-Bertani
LPS	lipopolysaccharide
MAb	monoclonal antibody
OD	optical density
PAI	*Pseudomonas aeruginosa* autoinducer
PAI-1	*N*-(3-oxododecanoyl)-L-homoserine lactone
PAI-2	*N*-butanoyl-L-homoserine lactone
PB	phosphate buffer
PBS	phosphate buffered saline

ppGpp	guanosine-tetraphosphate
PQS	2-heptyl-3-hydroxy-4-quinolone
RBD	receptor-binding domain
RNA	ribonucleic acid
RT	room temperature
SA	streptavidin
SD	standard deviation
SDS-PAGE	sodium docecyl suphate – polyacrylamide gel electrophoresis
SEM	standard error of the mean
spp.	species
TCP	toxin co-regulated pili
TSB	tryptic soy broth

Chapter 1

General Introduction

1. General Introduction

1. 1. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a γ-proteobacterium that is a member of the *Pseudomonadaceae* family of gram-negative bacteria, which also includes other opportunistic pathogens such as *Burkholderia* spp. and *Stenotrophomonas* spp. (Sedlak-Weinstein *et al.*, 2005). *P. aeruginosa* is a ubiquitous, non-fermenting, aerobic rod, that is found in diverse environments including soil and water, with the unique ability to infect many species of plants and animals (D'Argenio *et al.*, 2001; Green *et al.*, 1974; Pellett *et al.*, 1983; Plotnikova *et al.*, 2000). Although *P. aeruginosa* is strictly an aerobic bacterium, reports of growth under anaerobic conditions, particularly during biofilm growth in which nitrate, nitrite, or nitrous oxide substitute as the terminal electron acceptors have been reported (Davies *et al.*, 1989; Yoon *et al.*, 2002). *P. aerugionsa* is highly motile, and this motility is conferred by flagella and type IV pili, which are located at a single pole (Boone *et al.*, 2001; Bradley, 1980). Type IV pili are very important surface associated dynamic protein structures that are involved in a number of processes, including the establishment of an infection by *P. aeruginonsa* (Figure 1-1). The first scientific study published in 1882 on *P. aeruginosa* infections described the characteristic coloration of bandages, which was later discovered to be due to the production of blue-green secondary metabolites (Gessard, 1882; Grossowicz *et al.*, 1957).

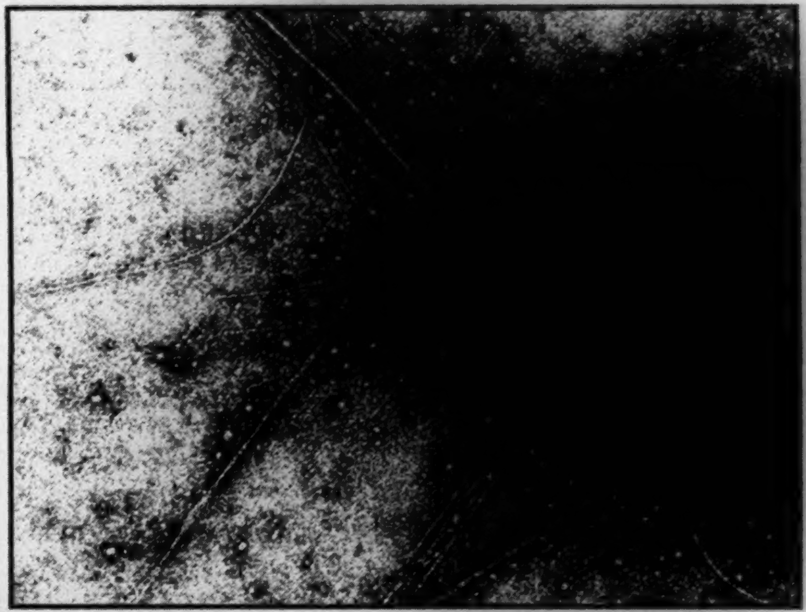


Figure 1-1. Transmission electron micrograph of *P. aeruginosa* strain K. Electron microscopy (EM) of PAK after staining with 1% aqueous molybate pH 7.0 using a Hitachi H-700i EM operating at an accelerating voltage of 75 kV. The arrow illustrates the polar location of the type IV pili on the surface of the *P. aeruginosa* bacterium. This figure was produced by Randy Irvin.

1. 2. *P. aeruginosa* infections

Today *P. aeruginosa* infections are a major concern in hospital environments as this bacterium is a common colonizer of hospitals and has even been isolated from cleaning solutions and tap water in association with outbreaks (Engelhart *et al.*, 2002; Ferroni *et al.*, 1998). As *P. aeruginosa* is an opportunistic pathogen that persists in hospital environments, this microbe is a major cause of hospital acquired infections including ventilator-associated pneumonia, urinary tract infections and bacteremia (Bagshaw and Laupland, 2006; Chastre and Fagon, 2002; Rossolini and Mantengoli, 2005; Shaw, 2005). The above mentioned infections also occur in immunocompromised individuals including HIV and neutropenic cancer pateints (Lau *et al.*, 2005; Manfredi *et al.*, 2000). Furthermore, the ability of *P. aeruginosa* to colonize abiotic surfaces including stainless steel is a major problem in hospital burn units, as bacterial wound infections resulting in sepsis are the major cause of death in this population (Giltner *et al.*, 2006; Pruitt *et al.*, 1998). *P. aeruginosa* infections do not always occur in hospital environments, as mild, self-limiting infections can occur in healthy individuals. Colonization of other abiotic surfaces such as extended wear contact lenses often results in infection of the cornea, which can cause vision loss (Fleiszig and Evans, 2002). In addition, mild skin infections can occur after swimming or showering in contaminated water (Gustafson *et al.*, 1983). Therefore, *P. aeruginosa* is able to cause a number of infections in many different organs in the human host. The major problem with *P. aeruginosa* infections is the high innate and acquired antibiotic resistance of this organism, due in part to a large number of genetically encoded efflux pumps and the reduced permeability of the outer membrane (Hancock, 1997; Obritsch *et al.*, 2005; Poole, 2001; Stover *et al.*, 2000). These characteristics make treatment of diverse *P. aeruginosa* infections extremely difficult. Consequently, *P. aeruginosa* infections often result in high patient morbidity and mortality.

1. 3. *P. aeruginosa* chronic lung infections in cystic fibrosis patients

The most common chronic infection of *P. aeruginosa* occurs in the lungs of individuals suffering from the genetic disorder cystic fibrosis (CF) (Sadikot *et al.*, 2005). *P. aeruginosa* is the most prevalent bacteria isolated from the CF lung associated with chronic infection, and *P. aeruginosa* infections are responsible for most of the morbidity and mortality of CF patients (Hoiby *et al.*, 2005). Cystic fibrosis is an autosomal recessive disorder that is prevalent in Caucasian populations resulting from mutations in the CF transmembrane conductance regulator (CFTR) (Cutting, 2005). CFTR is a multi-domain protein found in the apical membrane that functions as a chloride channel and is expressed in many cell types, where mutations result in abnormal secretion in the lungs, intestine, and pancreas (Hanrahan and Wioland, 2004). There are a large number of mutations in the CFTR that result in CF, to date there are more than 1000 characterized mutations, although the most widely distributed mutation is ΔF508 (Rowntree and Harris, 2003). All CFTR mutations lead to an absence of functional protein in the apical membrane through transcriptional termination or misfolding of the mature protein (Boucher, 2004).

There are several potential reasons for why CF patients are more susceptible to lung infections with *P. aeruginosa* and other microbes. Increased viscosity and abnormal composition of airway secretions, results in poor clearance of invading microbes, in addition to decreased function of anti-bacterial peptides (Davies, 2002). Another potential reason for the augmented colonization of CF lungs is an increased amount of asialo-GM$_1$ on the surface of epithelial cells, which is a specific cellular receptor for *P. aeruginosa* (Bryan *et al.*, 1998; Lee *et al.*, 1994; Saiman and Prince, 1993). The increased number of surface receptors may facilitate initial colonization, however, later during chronic infections *P. aeruginosa* is not actually attached to the airway epithelium (Prince, 2002; Schroeder *et al.*, 2001; Singh *et al.*, 2000). It has also been suggested that CFTR itself functions in an innate immune response, through uptake and removal of invading *P. aeruginosa* (Pier *et al.*, 1996). Therefore, mutations that result in non-functional CFTR would prevent this innate bacterial clearance mechanism (Pier *et al.*, 1996).

New research suggests that individuals are colonized with *P. aeruginosa* as early as 3 years of age (Burns *et al.*, 2001). Early colonization is normally followed by periods of eradication due to intensive antimicrobial treatment, but eventually the individual develops a chronic *P. aeruginosa* pulmonary infection (Hoiby *et al.*, 2005). Chronic infections are associated with specific changes in *P. aeruginosa*. During the establishment of a chronic infection, *P. aeruginosa* changes from a planktonic, motile, non-mucoid organism into a sessile, non-motile, mucoid organism (Sadikot *et al.*, 2005).

The mucoid phenotype is due to the production of alginate, an extracellular capsule like polysaccharide composed of D-mannuronic acid and L-guluronic acid (Linker and Jones, 1966). Interestingly, environmental isolates of *P. aeruginosa* do not overproduce alginate and conversion to this phenotype is associated with mutations in the *muc* loci (Govan and Deretic, 1996). Mutations in *mucA* or *mucB* lead to a deregulation of AlgT, an alternative sigma factor that controls production of the mucoid phenotype (Martin *et al.*, 1993a; Martin *et al.*, 1993b; Xie *et al.*, 1996). In this regard, a large number of clinical *P. aeruginosa* strains isolated from chronic CF infections are considered hypermutable, which may contribute to their persistence (Oliver *et al.*, 2000). The production of alginate is associated with biofilm formation, which is another important characteristic of chronic *P. aeruginosa* infections (Hoiby *et al.*, 2001; Singh *et al.*, 2000). Formation of biofilms and alginate production by *P. aeruginosa* result in high resistance to host responses and antibiotic treatments (Govan and Deretic, 1996). Although alginate and biofilm formation are hallmarks of chronic CF infections, other virulence factors are probably involved in the initial colonization by *P. aeruginosa*. The initial colonization of the CF lung by *P. aeruginosa* may resemble the sequence of events that occurs during acute *P. aeruginosa* pulmonary infections.

1. 4. *P. aeruginosa* virulence factors

The ability of *P. aeruginosa* to cause a number of different infections results from the large genome encoding a variety of diverse virulence factors (Stover *et* *al.*, 2000). Interestingly, new high throughput methods have established that environmental and infectious strains of *P. aeruginosa* are remarkably similar, suggesting that all strains possess the genes necessary for survival in diverse environments (Goodman and Lory, 2004). Sequencing of the *P. aeruginosa* genome revealed a remarkably high number of putative transcriptional regulators representing ~9% of the genome (Stover *et al.*, 2000). This suggests that transcriptional control plays a major role in persistence, and ultimately the virulence of *P. aeruginosa* (Goodman and Lory, 2004). Consequently, the virulence factors will be described followed by an analysis of coordinate expression by transcriptional regulators.

The first step in the establishment of any infection is colonization, which requires adherence to host tissues (Beachey, 1981). Attachment to epithelial cells by *P. aeruginosa* is mediated by several adhesins including type IV pili, which account for most cellular binding (Doig *et al.*, 1988; Farinha *et al.*, 1994; Woods *et al.*, 1980), alginate (Doig *et al.*, 1987; Mai *et al.*, 1993), LPS (Gupta *et al.*, 1994; Zaidi *et al.*, 1996), Exoenzyme S (Baker *et al.*, 1991), Exotoxin A (Moller *et al.*, 1994), outer membrane protein F (Azghani *et al.*, 2002) and flagella (Feldman *et al.*, 1998). After colonization *P. aeruginosa* produces a multitude of virulence factors that alter host cells and aid in dissemination.

P. aeruginosa produces a number of extracellular products including toxins and hydrolytic enzymes, which act on host cells and other components in the extracellular matrix. The secreted proteases, including elastase and alkaline protease, contribute to the virulence of *P. aeruginosa* by tissue destruction and therefore dissemination (Pavlovskis and Wretlind, 1979; Tang *et al.*, 1996; Twining *et al.*, 1993). Elastase, which is capable of degrading several host proteins including collagen and laminin, breaks down the extracellular matrix and allows for dissemination (Bejarano *et al.*, 1989; Heck *et al.*, 1986a; Heck *et al.*, 1986b). In addition, elastase degrades immunity proteins such as IgA, surfactants, and complement, which allows *P. aerugionsa* to persist during an immune response (Alcorn and Wright, 2004; Mariencheck *et al.*, 2003; Wretlind and Pavlovskis, 1983). The actions of elastase are mirrored by alkaline protease on fibrin and components of the immune system to produce similar results (Sadikot *et al.*, 2005). *P. aeruginosa* produce another secreted enzyme exotoxin A, however, this enzyme acts inside a host cell after induction of receptor mediated endocytosis (Fitzgerald *et al.*, 1980; Kounnas *et al.*, 1992). Once inside host cells exotoxin A ADP-ribosylates elongation factor-2 leading to an inhibition of protein synthesis and eventually cell death (Jorgensen *et al.*, 2005). Another secreted product, which lacks enzymatic activity, is pyocyanin a blue-green cytotoxic pigment that often discolors bandages from infected wounds (Lau *et al.*, 2004). Pyocyanin causes a number of effects, including inhibition of ciliary function (Sorensen and Klinger, 1987), alteration of calcium homeostasis (Denning *et al.*, 1998a), and immune modulation (Denning *et al.*, 1998b; Denning *et al.*, 2003). Furthermore, there are other secreted enzymes and metabolites that produce similar results to those described above and therefore will not be discussed.

In addition to the many extra-cellular products that contribute to the pathogenesis of this microbe, *P. aeruginosa* also produces a type III secretion system, which injects enzymatic proteins directly into host cells that effect their physiology (Mota *et al.*, 2005). The *P. aeruginosa* type III secretion apparatus is a multiple protein complex that acts like a needle to deliver four effector molecules into host cells ExoS, ExoT, ExoU, and ExoY (Frank, 1997; Lyczak *et al.*, 2000a). The type III secretion system mediates killing of polymorphonuclear phagocytes, marcrophages, and epithelial cells (Dacheux *et al.*, 1999; Hauser and Engel, 1999). Type IV pili are also involved in this process as cellular binding is essential, and retraction may precede type III secretion (Comolli *et al.*, 1999a; Comolli *et al.*, 1999b; Sundin *et al.*, 2002). Production of these effector proteins is dependent on host cell contact and other environmental conditions (Hornef *et al.*, 2000; Vallis *et al.*, 1999). ExoS and ExoT are homologous proteins with two functions: a carboxy-terminal ADP-ribosylation, and amino-terminal GAP activity (Barbieri, 2000; Garrity-Ryan *et al.*, 2000; Goehring *et al.*, 1999; Krall *et al.*, 2000). These enzymatic activities result in cytoskeletal rearrangements and inhibit phagocytosis (Barbieri, 2000; Sun and Barbieri, 2003). ExoU is a cytotoxic phospholipase (Sato and Frank, 2004) and ExoY is an adenylate cyclase (Yahr *et al.*, 1998). These effector proteins modulate host cell functions resulting in tissue damage.

Therefore, after colonization *P. aeruginosa* releases a number of proteins that aid in dissemination, including proteins that are directly injected into host cells to modulate their functions. All of these virulence activities are coordinately regulated by several transcriptional activators.

1. 5. Global regulation of virulence

Although there are many levels to the genetic regulation of *P. aeruginosa* virulence factors, the focus of the following discussion will be on global regulation. Therefore, quorum-sensing will be discussed in detail, including regulation of quorum-sensing by other global regulation systems. Quorum-sensing is a cell density dependent mechanism that was first described in *Vibrio fischeri* in the 1970s and controls autoinduction of luminescence (Nealson *et al.*, 1970). The system works by sensing the concentration of a synthesized cell permeable autoinducer produced by *V. fischeri* (Kaplan and Greenberg, 1985). At high cell densities the autoinducer is produced at sufficient concentrations to activate the *lux* genes (Fuqua *et al.*, 1994). All quorum-sensing systems require two components: a LuxI like protein, which is the cytoplasmic enzyme responsible for synthesis of an acylated homoserine lactone and a LuxR like protein, which interacts with the acylated homoserine lactone to activate promoters of genes regulated by quorum-sensing (Venturi, 2006b). *P. aeruginosa* has several quorum-sensing systems, all of which are involved in the regulation of virulence (de Kievit and Iglewski, 2000). Quroum-sensing mutants of *P. aeruginosa* are attenuated for virulence in both the burnt and acute pneumonia mouse models of infection (Pearson *et al.*, 2000; Rumbaugh *et al.*, 1999), indicating that this type of genetic regulation is important for pathogenesis.

There are two closely linked acylhomoserine lactone quorum-sensing systems in *P. aeruginosa* involved in the regulation of virulence factors, the *las* and *rhl* systems (Gambello and Iglewski, 1991a; Ochsner *et al.*, 1994). These two systems control many extracellular virulence determinants involved in host tissue damage and cellular modulation, which are not produced until a sufficient population of *P. aeruginosa* is present at the site of infection (Smith and Iglewski, 2003). LasR interacts with *N*-(3-oxododecanoyl)-L-homoserine lactone, produced by LasI, to alter genetic regulation of genes, including those that produce elastase and exotoxin A (Figure 1-2-A) (Gambello and Iglewski, 1991a; Toder *et al.*, 1991). Interaction with *N*-(3-oxododecanoyl)-L-homoserine lactone causes multimerization of LasR, which is required for transcriptional activation (Kiratisin *et al.*, 2002). The *las* system can also regulate the *rhl* system, where RhlR interacts with *N*-butanoyl-L-homoserine lactone produced by RhlI to regulate production of gene products, including pyocyanin and siderophores (Figure 1-2-B) (Latifi *et al.*, 1995a; Latifi *et al.*, 1996; Winson *et al.*, 1995b). RhlR is found as a homodimer in the absence of *N*-butanoyl-L-homoserine lactone, and *N*-(3-oxododecanoyl)-L-homoserine lactone causes dissociation of these dimers (Ventre *et al.*, 2003). Furthermore, a previous report demonstrated that *N*-(3-oxododecanoyl)-L-homoserine lactone prevents the interaction of RhlR with *N*-butanoyl-L-homoserine lactone, which stops the transcriptional activation of RhlR controlled genes (Pesci *et al.*, 1997). This demonstrates another point of interplay between these systems at the post-translational level. A third quorum-sensing like system, *pqs*, requires an intercellular signaling molecule 2-heptyl-3-hydroxy-4-quinolone (PQS) (Figure 1-2-C) (Pesci *et al.*, 1999). Production of PQS is regulated by LasR, and controls the expression of some RhlR regulated genes, which provides a link between the *las* and *rhl* systems (Deziel *et al.*, 2004;

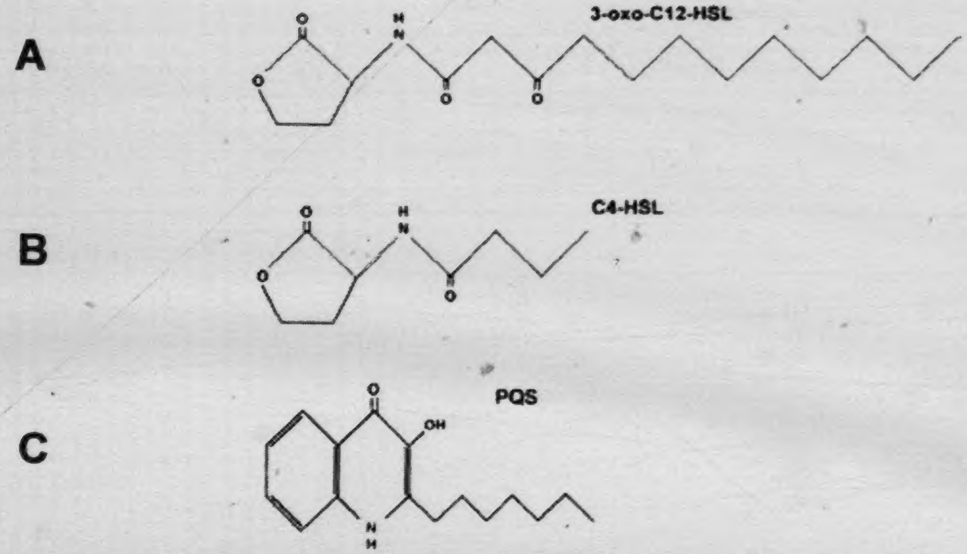


Figure 1-2. The structure of the quorum sensing autoinducers expressed by *P. aeruginosa*. (A) The structure of *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), which is synthesized by LasI and interacts with LasR. (B) The structure of *N*-butanoyl-L-homoserine lactone (C4-HSL), which is synthesized by RhlI and interacts with RhlR. (C) The structure of 2-heptyl-3-hydroxy-4-quinolone (PQS), which is synthesized by two gene operons *phnAB* and *pqsABCDE* and has no known cognate transcriptional response regulator. This figure was adapted from a review article (Juhnas *et al*., 2005).

Diggle *et al.*, 2003; McKnight *et al.*, 2000). Consequently, mutations in the *pqs* system result in a decrease in virulence of *P. aeruginosa* (Cao *et al.*, 2001). Furthermore, there is a LuxR homologue QscR with no cognate acylhomoserine lactone synthase that is involved in the quorum-sensing response and virulence (Chugani *et al.*, 2001). Mutations of *qscR* result in a hypervirulent phenotype, and recent studies indicate that QscR delays expression of some genes that are regulated by LasR and RhlR (Chugani *et al.*, 2001). This may be a result of the ability of QscR to form heterodimers with LasR or RhlR (Ledgham *et al.*, 2003). Furthermore, QscR also interacts with *N*-(3-oxododecanoyl)-L-homoserine lactone to regulate genes outside of the quorum sensing regulon, which are not regulated by either LasR or RhlR (Lee *et al.*, 2006; Lequette *et al.*, 2006).

Together these intimately linked systems control a large percentage of the genome, including many genes that are not involved in pathogenesis (Schuster *et al.*, 2003; Wagner *et al.*, 2003; Whiteley *et al.*, 1999). Quorum-sensing is regulated by several other global regulators in addition to being cell density dependent (Figure 1-3). The realization that quorum-sensing is not an independent system came from the observation that addition of acylhomoserine lactones to *P. aeruginosa* cells does not produce an effect until stationary phase is reached (Diggle *et al.*, 2002; Whiteley *et al.*, 1999; Winzer *et al.*, 2000). Interestingly, there is evidence that RpoS, the stationary phase sigma factor, may directly repress the transcription of *rhlI*, which provides a partial explanation for the delayed response to exogenous acylhomoserine lactones (Whiteley *et al.*, 2000). However, there is also evidence that RpoS directly activates and

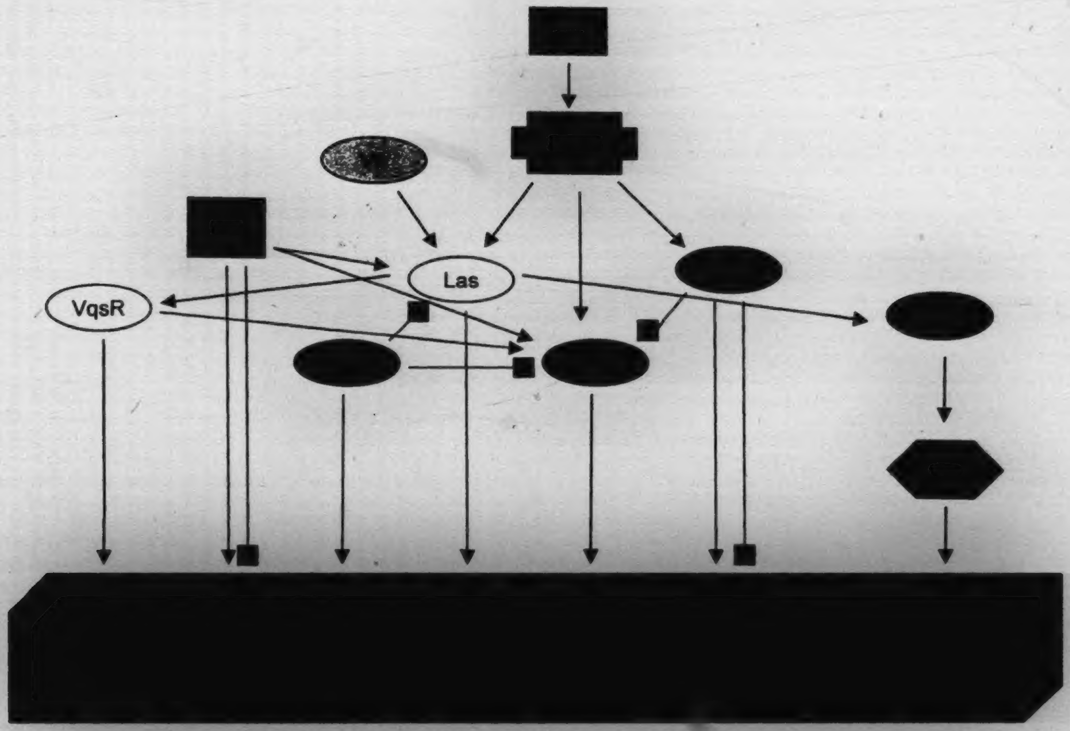


Figure 1-3. A simplified schematic diagram representing the global control of the quorum sensing regulon. The quorum sensing cell density regulatory pathways are Las, Rhl, and PQS. All of the other regulatory factors belong to global regulation pathways. Refer to section 1-5 for an in depth explanation of the intercommunication between regulatory pathways. The arrows represent positive control and the boxes represent negative control. This figure was reproduced and adapted from a review article (Schuster and Greenberg, 2006).

represses quorum-sensing genes (Schuster *et al.*, 2004).

Other global regulators also participate in the quorum-sensing response, such as Vfr, a homologue of the *E. coli* catabolite repressor CRP, which responds to cAMP, and directly induces *lasR* transcription (Albus *et al.*, 1997). In addition, Vfr is also involved in general virulence regulation of the type III secretion system, type IV pilus production, and twitching motility (Beatson *et al.*, 2002a; Wolfgang *et al.*, 2003). Over-expression of the stringent response protein RelA, which synthesizes ppGpp under starvation conditions, and ppGpp leads to early induction of some quorum-sensing regulated genes (van Delden *et al.*, 2001). The classic two component regulatory system GacA/GacS, and the transcriptional regulators VqsR and MvfR, are also involved in quorum-sensing control (Deziel *et al.*, 2005; Reimmann *et al.*, 1997; Wagner *et al.*, 2003). Therefore, control of virulence by quorum-sensing is very complex and utilizes multiple levels of control to globally regulate the cell density dependent pathways.

Another important genetic control system is required to control the switch from acute to chronic infection (Goodman *et al.*, 2004). Acute infection requires the expression of various virulence factors associated with cytotoxic effects such as the type III secretion system (Sawa *et al.*, 1999). However, these genes are not required for chronic infection and are suppressed, while genes required for production of the exopolysaccharide biofilm matrix such as *psl* and *pel* gene clusters are activated (Friedman and Kolter, 2004a, b; Matsukawa and Greenberg, 2004a). This switch is controlled in part by RetS and LadS, which are both hybrid sensor kinase response regulators that act in response to environmental signals (Figure 1-4) (Goodman *et al.*, 2004; Ventre *et al.*, 2006). RetS activates genes involved in acute infections, whereas LadS activates genes involved in chronic infections (Goodman *et al.*, 2004; Ventre *et al.*, 2006). Both of these systems act upstream of GacA/S, and therefore add another level of complexity to the genetic control of virulence in *P. aeruginosa* (Goodman *et al.*, 2004; Ventre *et al.*, 2006). Future research is needed to expand the picture on global regulatory networks that control virulence in both acute and chronic infections.

1. 6. Type IV pili

Type IV pili are polar, filamentous protein structures, which are produced on both pathogenic and environmental species of gram-negative bacteria, and mediate a number of important microbial processes (Figure 1-1). Many well characterized human pathogens produce type IV pili including enteropathogenic *Escherichia coli* (EPEC) (Giron *et al.*, 1991), pathogenic *Neisseria* spp. (Swanson, 1973), *Vibrio cholerae* (Taylor *et al.*, 1987), and the opportunistic pathogen *Pseudomonas aeruginosa* (Weiss, 1971). In addition, type IV pili are produced by animal pathogens such as *Dichelobacter nodosus* (Elleman, 1988) and *Moraxella bovis* (Jayappa and Lehr, 1986), the plant pathogen *Xylella fastidiosa* (Meng *et al.*, 2005), and pathogens capable of infecting both humans and animals, for example *Francisella tularensis* (Gil *et al.*, 2004). This indicates that type IV pili are major virulence factors for a diverse group of bacteria.

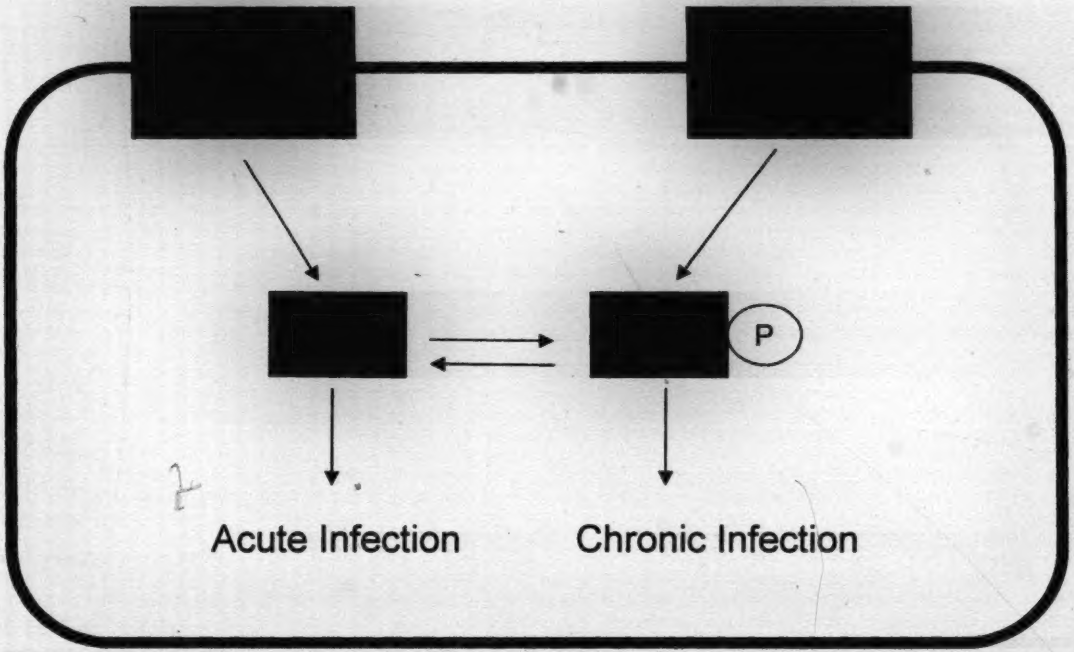

P
Acute Infection
Chronic Infection

Figure 1-4. A simplified schematic diagram depicting the switch between acute and chronic infection. Both RetS and LadS act upstream of GacA to mediate the switch between chronic and acute infection. See section 1-5 for for an in depth explanation. This figure was reproduced and adapted from Ventre *et al.* (2006).

Mutations that prevent the production of pili in any of the above pathogens cause significant reductions in virulence (Bieber *et al.*, 1998; Forslund *et al.*, 2006; Herrington *et al.*, 1988; Kennan *et al.*, 2001b; Meng *et al.*, 2005; Ruehl *et al.*, 1988; Sato *et al.*, 1988; Swanson *et al.*, 1987; Tang *et al.*, 1995).

However, the type IV pili of some bacteria do not contribute to virulence and are probably required for persistence in the natural environment. For example, *Legionella pneumophila* produces type IV pili that adhere to human epithelial cells, macrophages, and the protozoan *Acanthamoeba polyphaga* (Stone and Abu Kwaik, 1998). Although *L. pneumophila* type IV pili are adherence factors, pilus-deficient mutants are not impaired in their ability to colonize mouse lungs (Rossier *et al.*, 2004).

Binding to host cells is a common function of type IV pili and is intimately linked to the pathogenesis of many microbes. The bundle forming pili of enteropathogenic *E. coli* mediate adherence to the intestinal epithelium and microcolony formation (Donnenberg *et al.*, 1992). Similar results have been obtained by studying the toxin co-regulated pilus of *V. cholerae*, although recent data suggests that epithelial cell binding and microcolony functions of the pilus are located on independent domains of the intact fibers (Kirn *et al.*, 2000). *P. aeruginosa* pili can bind to both Buccal epithelial and tracheal epithelial cells (Doig *et al.*, 1988; Woods *et al.*, 1980). Furthermore, *P. aeruginosa* type IV pili are involved in formation of microcolonies *in vitro* (Klausen *et al.*, 2003b; O'Toole and Kolter, 1998), which may be important for biofilm formation during chronic infections (Singh *et al.*, 2000). *N. gonorrhoeae* type IV pili adhere to the epithelium of the urogenitial tract through two different pilus receptor binding domains (Rudel *et al.*, 1992). *Salmonella enterica* serovar Typhi produce type IV pili that mediate adherence and uptake by the intestinal epithelium, although the contributions to pathogenesis are currently unknown (Zhang *et al.*, 2000). Therefore, the type IV pili from all the above pathogens mediate adherence to a variety of host epithelial cells, which results in a diverse number of infections in different locations within the human host.

Production of type IV pili by the environmental microbe *Myxococcus xanthus* results in adherence to abiotic substrates and a unique form of community movement termed social motility (Wu and Kaiser, 1995). Social motility is equivalent to twitching motility, which is a common function of type IV pili that requires repeating cycles of pilus extension and retraction (Mattick, 2002). The cyanobacterium *Synechocystis* sp. PCC6803 produces type IV pili and is capable of twitching motility (Bhaya *et al.*, 2000). Therefore adherence in general is a common function of type IV pili, which enables some bacteria to travel across surfaces by twitching motility.

Another mode of adherence is required for bacterial conjugation, which is the ability to transfer plasmid from donor to recipient cells and is a unique bacterial process (Llosa *et al.*, 2002). Interestingly, the conjugative plasmid R64 from the incompatibility group I1 produces type IV pili that are required for mating in liquids (Kim and Komano, 1997). These conjugative pili adhere to LPS in the donor cell (Ishiwa and Komano, 2000), providing another example of an adherence function for type IV pili. Although R64 produces type IV pili conjugation occurs using a type IV secretion system that encodes an alternative class of pili, which are thicker and more rigid (Bradley, 1983; Lawley *et al.*, 2003). Once cell to cell contact is made, the conjugation pilus retracts to bring the cells in close contact, which is necessary for plasmid transfer through the mating channel (Christie, 2001). Type IV secretion systems are able to transport both DNA and/or proteins and span the bacterial cell envelop (Lawley *et al.*, 2003). Therefore, type IV secretion system can mediate effector translocation, DNA uptake and release, and conjugative transfer (Kostakioti *et al.*, 2005). These systems are made up of transfer genes and membrane complex genes. Type IV secretion systems can be cell-contact dependent or cell-contact independent processes (Kostakioti *et al.*, 2005). Therefore, type IV secretion can be similar to type III serection and mediate the transfer of proteins or DNA from the donor directly into the cytoplasm of the recipient. Type II, III, IV secretion systems require common ancestrally conserved ATPases that are required to produce the energy that support these processes (Kostakioti *et al.*, 2005).

Type IV pili can also be receptors for bacteriophage, which are bacteria specific viruses. There are several type IV pilus specific phages of *P. aeruginosa* including PO4, which is an icosahedral RNA phage with a long non-contractile tail (Bradley, 1973). Interestingly, infection with PO4 requires functional type IV pili, as retraction deficient mutants are resistant to phage infection (Bradley, 1974). The ability of type IV pili to act as receptors for bacteriophage also contributes to the pathogenicity of some species. In fact, acquisition of the genes required for cholera toxin production in *V. cholerae* resulted from infection with the pilus specific CTX bacteriophage (Waldor and Mekalanos, 1996).

Therefore, type IV pili are produced by a diverse number of gram-negative bacteria that colonize a number of different environments and perform specific related functions necessary for survival. In addition, acquisition of novel genetic determinants can occur by production of functional type IV pili through horizontal or lateral gene transfer mediated by conjugation or bacteriophage infection.

1. 7. Structure of type IV pilins

All type IV pili examined to date are composed of 500 to 1000 copies of the major pilus subunit termed pilin, which are assembled to form surface associated fibers (Craig *et al.*, 2004; Strom and Lory, 1993). Several pilin structures have been solved by either X-ray crystallography or NMR. Type IV pilins are classified into two subgroups, type IVa and type IVb, based on differences in the length of the leader peptide and the size of the mature protein (Craig *et al.*, 2004). Structural studies on type IV pilin from both groups reveal similarities and differences between the major pilin subunits (Figure 1-5 and 1-6).

Members of the type IVa group include *pilA* from *P. aeruginosa* (Koga *et al.*, 1993) and *pilE* from *N. gonnorhoeae* (Perry et al., 1987). The type IVa pilins have an average length of 150 amino acids and have highly conserved N-terminal hydrophobic sequences (Craig *et al.*, 2004; Hazes *et al.*, 2000). Sequence conservation in the rest of the pilin is marginal at best, even between strains of the same species, such as *P. aeruginosa* strains, which share very low sequence identity (Audette *et al.*, 2004a). The hydrophobic N-terminal sequence

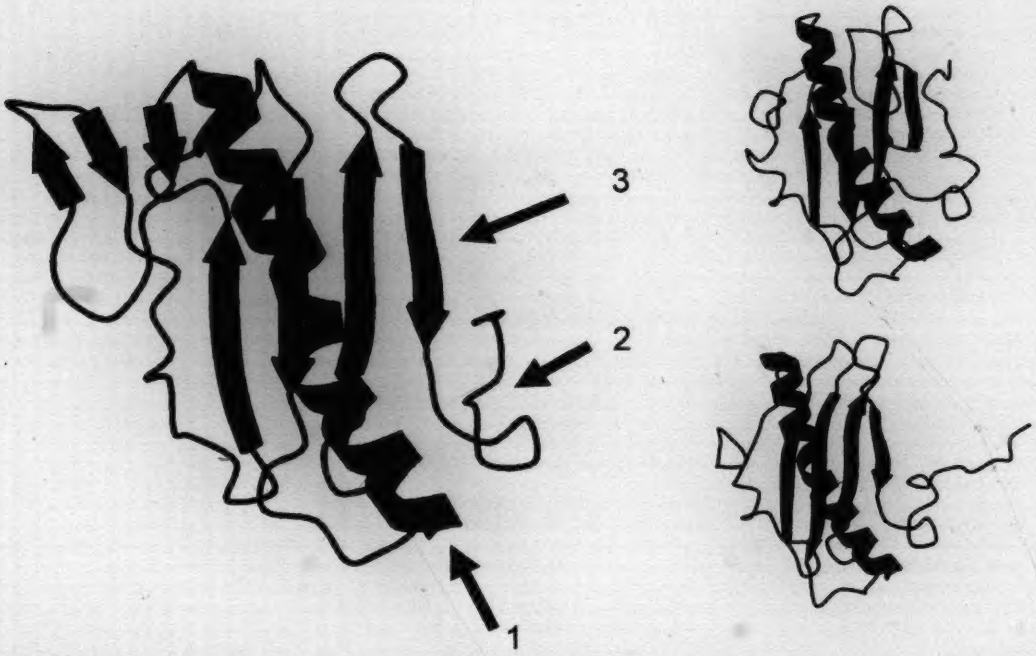


Figure 1-5. The structures of the type IVa N-terminal truncated pilins. The structural diagrams were created using Molscript. The red diagram is the *P. aeruginosa* PAK pilin structure (Hazes *et al.*, 2000). The orange diagram is the *N. gonorrhoeae* MS11 pilin structure (Parge *et al.*, 1995). The blue diagram is the *P. aeruginosa* K122-4 pilin structure (Audette *et al.*, 2004a). Arrow number one shows the N-terminal truncated α-helix domain. Arrow number two shows the C-terminal disulfide bonded loop, the RBD, or the D region. Arrow number three shows the 4-stranded anti-parallel β-sheet domain.

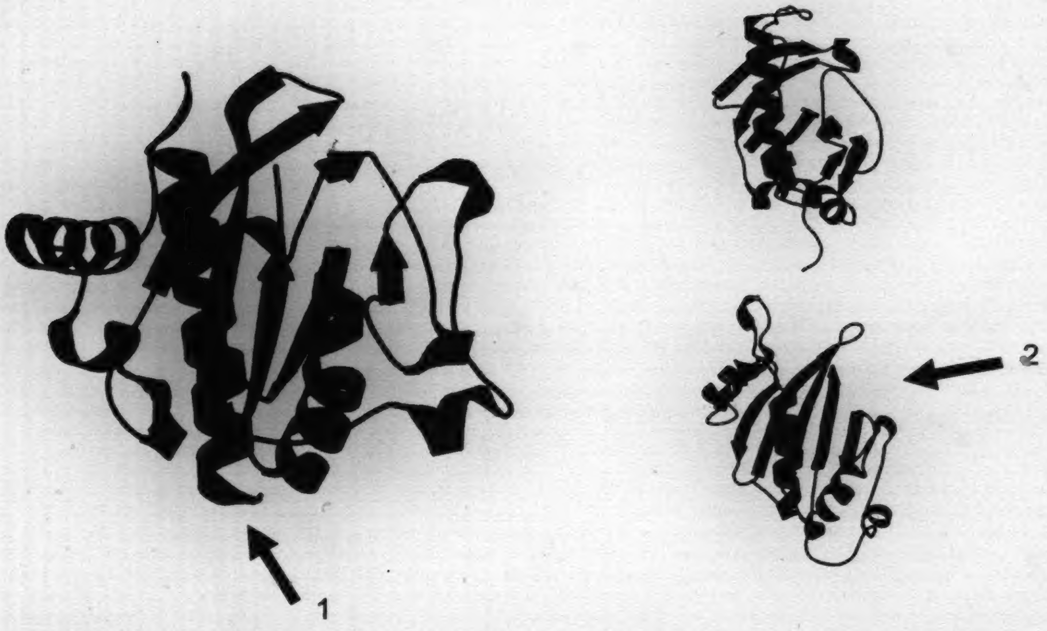


Figure 1-6. The structures of the type IVb N-terminal truncated pilins. The structural diagrams were created using Molscript. The green diagram is the *V. cholerae* TcpA pilin structure (Craig *et al.*, 2003). The cyan diagram is the EPEC BfpA pilin structure (Ramboarina *et al.*, 2006). The purple diagram is the *Salmonella enterica* serovar Typhi PilS structure (Xu *et al.*, 2004). Arrow number one shows the N-terminal truncated α-helix domain. Arrow number two shows the anti-parallel β-sheet domain with a variable number of β-strands. The D-region is not pointed out for the type IVb structures as this region is much larger.

forms a long α-helix (Craig *et al.*, 2003; Parge *et al.*, 1995). The rest of the pilin forms a globular head domain composed primarily of a 4-stranded anti-parallel β-sheet that lies on top of the α-helix (Audette *et al.*, 2004a; Hazes *et al.*, 2000; Parge *et al.*, 1995). A variable structure connects the α-helix to the β-sheet termed the αβ loop. For the *P. aeruginosa* PAK pilin, a small β-sheet connects these two structures (Craig *et al.*, 2003; Hazes *et al.*, 2000), whereas, for *P. aeruginosa* K122-4 pilin (Audette *et al.*, 2004a; Keizer *et al.*, 2001) this structure is a loop, and for *N. gonorrhoeae* MS11 pilin this variable region is a single helical turn (Parge *et al.*, 1995). A fourth structurally conserved portion of the pilins is the C-terminal disulfide bonded loop, or D region, although there is minor sequence conservation in this region (Hazes *et al.*, 2000). Even though the 12-29 residue loop between the two disulfides is highly variable, this region forms a β-hairpin structure connected to a loop (Parge *et al.*, 1995) or β-turns connected to each other (Audette *et al.*, 2004a; Craig *et al.*, 2003; Hazes *et al.*, 2000). Some of the type IVa pilins can be post-translationally modified by glycosylation and/or phosphorylation. Many of the *P. aeruginosa* type IVa pilins are glycosylated (Kus *et al.*, 2004), including the 1244 pilin at the C-terminal serine residue (Castric *et al.*, 2001; Comer *et al.*, 2002). *Neisseria* spp. pilins can also be glycosylated at Ser63 and/or are phosphorylated at Ser68 (Parge *et al.*, 1995; Stimson *et al.*, 1995).

Members of the type IVb group include *bfpA* from enteropathogenic *E. coli* (Donnenberg et al., 1992), *pilS* from *Salmonella enterica* serovar Typhi (Zhang *et al.*, 2000), and *tcpA* from *V. cholerae* (Taylor et al., 1987). The type IVb pilins have less sequence conservation in their N-terminal sequences than the type IVa pilins, although the N-terminus still forms an extended α-helix (Craig *et al.*, 2004). As the type IVb pilins are larger than the type IVa pilins with an average length of 190 amino acids, the type IVb pilins have extra structural features (Craig *et al.*, 2004). The globular region of TcpA is composed of a 5-stranded β-sheet with extra structural elements in the connecting loops, including two extra α-helices (Craig *et al.*, 2003). Intriguingly, the globular region of PilS is composed of a 7-stranded β-sheet, although the structural topology is similar to TcpA (Xu *et al.*, 2004). In contrast, the globular domain of BfpA has a 7-stranded β-sheet, and the structural topology is distinct (Ramboarina *et al.*, 2005). Furthermore, the D regions of the type IVb pilins are very different from the type IVa pilins. The D region of TcpA is very large and connects a segment consisting of 65 residues (Craig *et al.*, 2003); this domain in PilS is only 36 residues (Xu *et al.*, 2004) and 49 residues for BfpA (Ramboarina *et al.*, 2005). Although the D regions of the type IVb pilins vary in length, there are some conserved structural features similar to the D region of the type IVa pilins. In addition, superimposition of the D regions from the three type IVb pilins reveal that several hydrophibic positions are conserved (Ramboarina *et al.*, 2005).

Although there are striking structural differences between the type IV pilins of different species, the pilin monomers are assembled by complex protein machinery resembling the type II secretion system consisting of over 40 gene products (Jacobs *et al.*, 2003; Peabody *et al.*, 2003). The resulting pilus fibers can be up to several micrometers long, are flexible and thin (50-80Å) (Craig *et* *al.*, 2003; Folkhard *et al.*, 1981). In addition, these fibers are very strong and can withstand stress forces of approximately 100 pN (Maier *et al.*, 2002; Merz *et al.*, 2000; Touhami *et al.*, 2006). Even though type IV pilus fibers are easily visualized using electron microscopy, determination of the exact assembly parameters has proven difficult. Consequently, there are several different models for the architecture of the assembled pilus fibers.

Early diffraction data with *P. aeruginosa* pilus fibers suggested there were five subunits per turn, a ~40Å pitch, a 62Å outer diameter, and the α-helices ran parallel with the fiber axis (Folkhard *et al.*, 1981; Watts *et al.*, 1983a). The first proposed model for type IVa pili using the solved structure of a pilin subunit was for *N. gonorrhoeae* pili, which was a right-handed 1-start helix model with five subunits per turn, a pitch of 41Å, and a diameter of ~60Å (Parge *et al.*, 1995). In this model the β-sheets make up the exterior of the pilus, while the α-helices oligomerize to form a coiled-coil bundle in the pilus core (Parge *et al.*, 1995). There are several studies using type IVa pili, which indicate that the N-terminal α-helices are required for oligomerization. Substitution of a lysine for the glutamate at position 5 of the mature pilin abolishes formation of pili, unless a wild type pilin is expressed *in trans*, which results in the formation of heterogeneous pili with an uncharacteristic morphology (Pasloske *et al.*, 1989). This suggests that mutations in the conserved N-terminal α-helix abolish pili formation. The requirement of detergent to disassociate pili also suggests that the hydrobobic helices are important for oligomerization (Parge *et al.*, 1995; Watts *et al.*, 1982). The most compelling data suggesting that the N-terminal α-helices are required for pilus assembly, is that removal of this domain results in the formation of soluble pilin monomers (Audette *et al.*, 2004a; Hazes *et al.*, 2000; Keizer *et al.*, 2001). In addition, a recent study determined that addition of a hydrophobe to soluble K122-4 monomers causes oligomerization into a structure that highly resembles type IV pili (Audette *et al.*, 2004b).

A similar model for the assembled pilus was proposed using the structure of the truncated PAK pilin monomer, taking into consideration the biological function of *P. aeruginosa* pili (Hazes *et al.*, 2000). This model is based on the same assembly parameters used by Parge and colleagues (1995), and as such was a 1-start right-handed helix with five subunits per turn (Hazes *et al.*, 2000). However, this model proposed an inverted polarity of the pilus, where the α-helices would be exposed at the tip of the pilus as opposed to being buried in the bacterial membrane (Hazes *et al.*, 2000; Parge *et al.*, 1995). This polarity would occlude the C-terminal receptor binding domain along the length of the pilus to only expose this domain at the tip of the pilus in agreement with previous studies (Hazes *et al.*, 2000; Lee *et al.*, 1994). However, the C-terminal disulfide bonded loop would be exposed along the length of the pilus in the proposed model for *N. gonorrhoeae* pili with the α-helices buried in the membrane (Parge *et al.*, 1995). The polarity and surface exposure agrees with antibody data that suggest residues 140-159 of the C-terminal disulfide bonded loop are exposed on the surface of the *N. gonorrhoeae* pilus (Forest *et al.*, 1996). Although the above models for *P. aeruginosa* and *N. gonorrhoeae* pili are nearly identical, new differences in the fiber diffraction patterns suggest that there are some variations in the assembly of PAK and MS11 pili (Marvin *et al.*, 2003). A slightly different model was then proposed taking into account the structural information obtained using x-ray crystallography, NMR, and electrostatic surface complementarities between the pilin monomers (Keizer *et al.*, 2001). Using the truncated K122-4 pilin NMR solved structure, a 1-start left-handed model with five subunits per turn, an outer diameter of ~52Å, and a pitch of 41Å was proposed (Keizer *et al.*, 2001). Other variations of type IVa models have also been proposed including a right-handed 1-start helix with four subunits per turn and a left-handed 3-start helix with four subunits per turn for *P. aeruginosa* pili, where interactions between PAK pilin subunits occur in the αβ-loop of one subunit and the D-region of the neighbouring subunit (Craig *et al.*, 2004) (Figure 1-7).

Electron micrographs of the TCP pilus of the type IVb group suggest the pilus is a 3-start helical assembly with a 45 Å pitch and an approximate diameter of 80 Å (Craig *et al.*, 2003). A model was then constructed using both the EM fiber data, and the solved structure of the truncated TcpA pilin, corresponding to a left-handed 3-start helix with six subunits per turn (Craig *et al.*, 2003). The surface of the fiber is composed of the αβ-loop, the β-sheet, and the D-region (Craig *et al.*, 2003), and interactions between neighbouring TcpA pilin subunits occur between the αβ-loop and the D-region (Craig *et al.*, 2004). This model has also been proposed based on the NMR solved truncated PilS structure, with slightly different interactions between the αβ-loop and D-region between subunits likely due to additional structural elements in the αβ-loop, and therefore this model has a pitch of ~30 Å and outer diameter of ~100 Å (Xu *et al.*, 2004). Electron

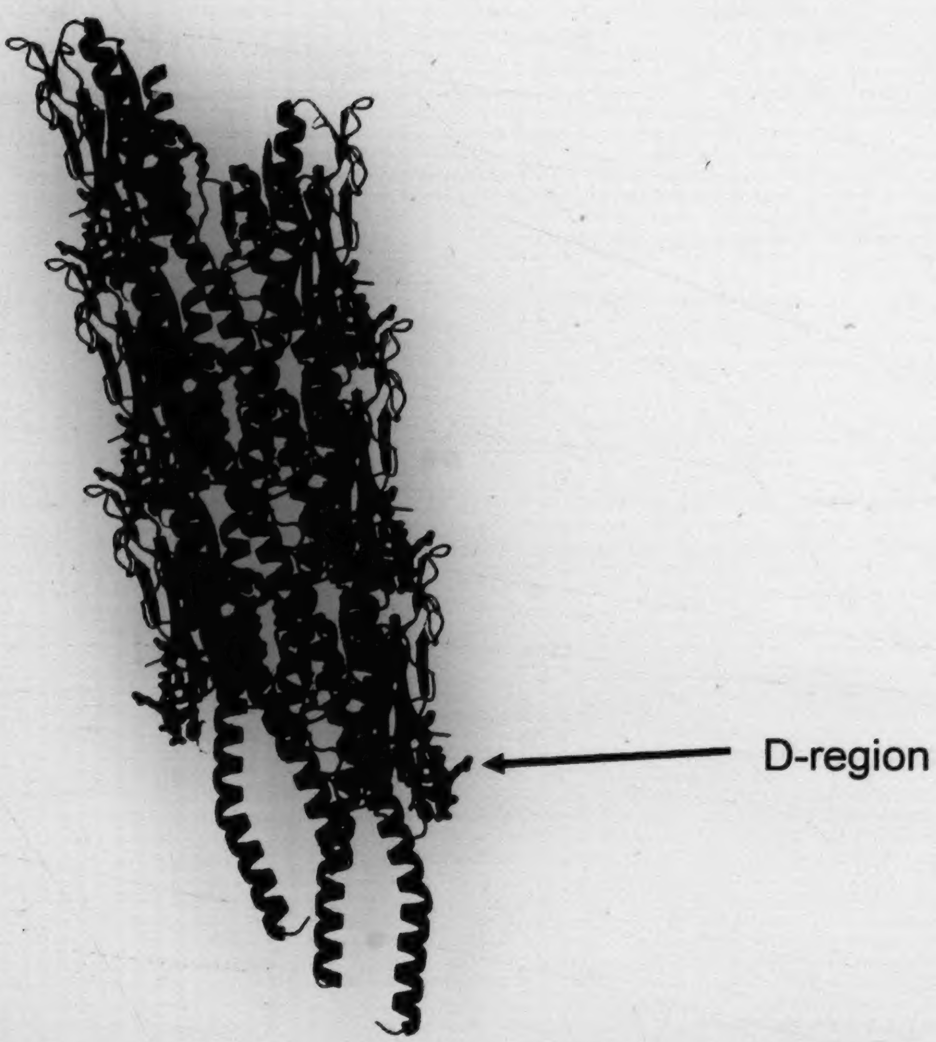


Figure 1-7. The 3-start left-handed hypothetical PAK pilus model with four subunits per turn proposed by Craig *et al.* (2004). The red regions represent the D-region or RBD that mediates binding to cellular receptors. The β-sheet domains form the exterior of the pilus and are solvent exposed along the length of the pilus. The N-terminal hydrophobic α-helices form the central core of the pilus and are hypothesized to act as an oligomerization domain. Adapted from a review (Burrows, 2005).

microscopy of the bundle forming pili of EPEC suggest a helical assembly with a diameter of ~75 Å and pitch of ~44 Å (Ramboarina *et al.*, 2005). The model proposed based on the EM and solved NMR structure of the truncated BfpA is a 3-start left-handed helix with six subunits per turn and similar interactions between the αβ-loop and the D-region to the above mentioned proposed structures for PilS and TCP (Craig *et al.*, 2003; Ramboarina *et al.*, 2005; Xu *et al.*, 2004). The new structural information on the type IVb pilins suggests a common architecture of a 3-stranded left handed helical assembly for all type IV pili where interactions occur through the αβ-loop and the D-region, but differ in the diameter, pitch, and number of subunits per turn. However, further research is required to fully elucidate the structure and assembly of the intact type IV pili from different species.

1. 8. Assembly of type IV pili

The above structural data has provided valuable insight into the functionality of type IV pili, although the biological assembly or biogenesis of type IV pili remains almost entirely a mystery. Even though the structural studies suggest there might be different assemblies of type IV pilus fibers, the complex protein machinery used for assembly is fairly conserved. This conservation is demonstrated by the ability to form functional pili after transfer of pilins from one species into another (Beard *et al.*, 1990; Elleman and Peterson, 1987; Mattick *et al.*, 1987; Watson *et al.*, 1996). The type IV pilin genes are normally found on the bacterial chromosome, with some exceptions such as *bfpA*, which is encoded on the 92 kb virulence associated plasmid (Giron *et al.*, 1991, 1993). In addition, the genes for type IV pili expression can be found on large pathogenicity islands in some bacterial species (Brown and Taylor, 1995; Pickard *et al.*, 2003). The pilin genes are clustered with some pilus biogenesis genes, but are often found in close proximity to genes that are not involved in pilus assembly (Strom and Lory, 1993). The only protein with a well defined, required function, for type IV pili biogenesis is PilD from *P. aeruginosa* and homologues from other species (Johnston *et al.*, 1995; Nunn and Lory, 1991; Tonjum *et al.*, 1995).

The type IV pilins are synthesized as pre-pilins that must be post-translationally modified before assembly into pilus fibers (Mattick, 2002). PilD is the bifunctional enzyme that performs the post-translational modifications to produce mature pilins (Nunn and Lory, 1991; Strom *et al.*, 1993). Firstly, PilD is the endopeptidase responsible for cleavage of the N-terminal leader peptide sequence, which is normally 6-8 amino acid residues long in type IVa pilins and 15-30 amino acid residues long in type IVb pilins with a net basic charge and a conserved glycine residue at position -1 relative to the cleavage site (Craig *et al.*, 2004; Nunn and Lory, 1991; Strom and Lory, 1992). Secondly, PilD is responsible for methylation of the new N-terminal amino acid, which is always a phenylalanine in the type IVa pilins, but which varies in the type IVb pilins (Craig *et al.*, 2004; Strom *et al.*, 1993). PilD homologues are necessary for pilus biogenesis and can complement *pilD* mutations in *P. aeruginosa* (de Groot *et al.*, 1994; Pepe *et al.*, 1996; Zhang *et al.*, 1994). Furthermore, PilD and its homologues are involved in the general secretion of proteins in many gram-negative bacteria via the type II secretion system; PilD was independently discovered and named XcpA (Bally *et al.*, 1991; Pugsley and Reyss, 1990).

Studies on type II secretion have focused on the pullulanase secretion by *Klebsiella* spp., which is well characterized, and has provided insight into the biogenesis of type IV pili (Pugsley *et al.*, 1997). As such, the biogenesis of type IV pili occurs through a type II secretion system, where PilD is responsible for the first processing step after Sec dependent secretion into the periplasm (Kostakioti *et al.*, 2005). Components of the type IV pili biogenesis system also have homologues to proteins involved in archaeal flagella biogenesis, and natural transformation systems of gram-positive bacteria, such as *Bacillus subtilis* (Dubnau, 1999; Hobbs and Mattick, 1993; Peabody *et al.*, 2003). All of these systems require several pre-pilin like proteins, a leader peptidase, nucleotide binding proteins, inner membrane proteins, and outer membrane proteins in the gram-negative bacteria (Hobbs and Mattick, 1993; Peabody *et al.*, 2003).

The steps after maturation of the pilins are poorly characterized, although the location and functions of some pilus biogenesis proteins have been described. Proteins that are involved in type IV pilus biogenesis in *P. aeruginosa*, *N. gonorrhoeae*, EPEC, and *V. cholerae* TCP are listed in table 1-1. Once the pre-pilin is processed, it must interact with the pilus assembly complex in the inner membrane, and therefore must be recruited to this protein machinery. Mutations in the cytoplasmic protein *pilB* or the integral inner membrane protein *pilC* of *P. aeruginosa* results in a bald or non-piliated phenotype (Koga *et al.*, 1993; Nunn *et al.*, 1990). PilB has a conserved ATP binding domain termed a Walker box

Table 1-1. Proteins required for type IV pilus biogenesis in several gram-negative bacterial species. This table was produced using information from (Russel, 1998) and PHI-BLAST analysis (Altschul *et al.*, 1997).

Domain Homologies*	*P. aeruginosa*	*N. gonorrhoeae*	EPEC	*V. cholerae*
Pilin	PilA	PilE	BfpA	TcpA
Secretin	PilQ	PilQ	BfpB	TcpC
ATP Motif	PilB	PilF	BfpD	TcpT
	PilT	PilT	BfpF	
	PilU	PilU		
Pre-pilin peptidase	PilD	PilD	BfpP	TcpJ
Sensor kinase	PilS			
Reponse regulator	PilR			
Pre-pilin cleavage site	PilE		BfpI	TcpB
	PilV			
	PilW			
	PilX			
	FimT			
	FimU			
			BfpJ	
			BfpK	
Pilin glycosylase	PilO			
CheY	PilG			
	PilH			
CheW	PilI			
CheR	PilK			
Unknown function		PilO		
	PilC	PilG	BfpE	TcpE
	PilP	PilP		
	PilM	PilM		
	PilN	PilN		
Pilus stabilization⁺	PilF	PilW		
Tip adhesion⁺	PilY1	PilC1		
Pilus stabilization⁺	PilY2	PilC2		
	PilZ			
			BfpC	
			BfpG	
			BfpH	
			BfpL	
			BfpU	

*Proteins in adjacent columns represent homologues

⁺These functions have only been demonstrated in *Neisseria* spp.

and mutations in this domain result in a non-piliated phenotype (Turner *et al.*, 1993). *P. aeruginosa* has two other cytoplasmic proteins, PilT and PilU that also contain Walker box motifs (Whitchurch *et al.*, 1991; Whitchurch and Mattick, 1994). Interestingly, mutations in either of these cytoplasmic proteins result in non-retractile pili and therefore a hyper-piliated phenotype (Whitchurch *et al.*, 1991; Whitchurch and Mattick, 1994).

Recent studies in EPEC have determined interactions between integral inner membrane proteins and cytoplasmic proteins that are involved in type IV pilus biogenesis (Crowther *et al.*, 2004). Interactions of BfpD with BfpE and BfpE with BfpF were demonstrated (Crowther *et al.*, 2004). In addition, all of the proteins self-associated indicating that these proteins function as multimers (Crowther *et al.*, 2004). BfpD forms homohexamers, potentially in a ring-like structure with a central pore, which is consistent with the structures of other members of this ATP binding family, including TrbB that functions during conjugation and is part of the type IV secretion apparatus from the RP4 conjugative plasmid (Krause *et al.*, 2000). Furthermore, it was demonstrated that BfpD interacts with two different sites on BfpE depending on whether BfpD has ATP or ADP bound (Crowther *et al.*, 2005). Interestingly, production of the cytoplasmic loop of BfpE in EPEC interacts with BfpF and prevents type IV pilus retraction, thereby producing a dominant negative effect (Crowther *et al.*, 2004). This data strongly suggest that BfpE, and its homologous integral membrane proteins sequester the ATP binding proteins BfpD and BfpF to transmit energy across the inner membrane to mediate pilus biogenesis (oligomerization), or pilus retraction (disassembly)

(Crowther *et al.*, 2004; Crowther *et al.*, 2005).

Studies on biogenesis of type IV pili in *N. gonorrhoeae* suggest that the pilus is formed in the periplasm before extrusion across the outer membrane (Wolfgang *et al.*, 2000). Mutations that prevent pilus retraction (*pilT*) and extrusion across the outer membrane (*pilQ*) result in the formation of pilus fibers that are sheathed in outer membrane, and cause reduced viability and growth rates (Wolfgang *et al.*, 2000). The actual components that mediate the oligomerization of pilins in the periplasm remain unknown.

The final component of this biogenesis system is the integral outer membrane protein termed a secretin, which is hypothesized to form a gated outer membrane channel (Bitter, 2003). Mutations in the pilus specific secretins of *P. aeruginosa*, *N. gonorrhoeae*, *N. meningitidis*, and EPEC result in a non-piliated phenotype (Drake and Koomey, 1995; Martin *et al.*, 1993c; Schmidt *et al.*, 2001; Tonjum *et al.*, 1998). Structural studies with the *N. meningitidis* PilQ complex have suggested that the secretin is a homododecameric structure composed of 12 PilQ proteins organized into a tetramer of trimers (Collins *et al.*, 2004). Furthermore, it was established that the secretin is a gated channel and that the pore is large enough to accommodate an assembled pilus (Collins *et al.*, 2004). Interestingly, purified type IV pili can interact with the secretin complex and structural changes occur in the secretin upon this interaction, suggesting that this interaction is dynamic (Collins *et al.*, 2005). Other pilus assembly components that are found in the outer membrane include PilC and PilW (Carbonnelle *et al.*, 2005; Rahman *et al.*, 1997). *Neisseria* spp. require an extra pilus component

PilC, which is localized at the pilus tip and is involved in stabilization of the fibers and cellular adherence (Rudel *et al.*, 1995b; Wolfgang *et al.*, 1998b). PilW has a similar function and is required for fiber stabilization, cellular adherence, and stabilizes PilQ multimers (Carbonnelle *et al.*, 2005). It remains to be determined which proteins, if any, are required for fiber stabilization and functionality in other organisms that produce type IV pili.

Taken together these data suggest that there are several steps to pilus biogenesis including translocation of pre-pilin across the inner membrane by the Sec system, processing of pre-pilin, assembly of pili in the periplasm, and extrusion through the outer membrane. Further research is required to determine the exact identity and function of all the proteins involved in this extremely complex process (Figure 1-8).

1. 9. Functions of type IV pili

As type IV pili are produced by such a wide variety of bacteria and are produced by complex conserved protein machinery, it is not surprising that type IV pili are involved in a number of important bacterial processes. These processes including colonization, twitching motility, natural transformation, and biofilm formation will be discussed in further detail below. Although there are many species of bacteria that utilize all of these pilus functions, the focus of the following section will be on the type IV pili from *P. aeruginosa*.

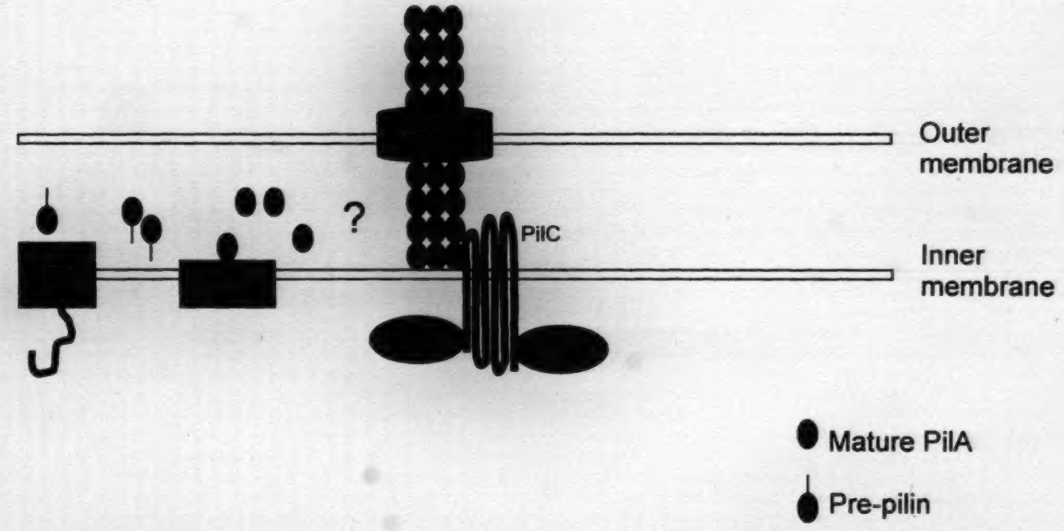


Figure 1-8. A simplified schematic of pilus assembly based on the results presented by Nunn and Lory (1991), Crowther and colleagues (2004), and Wolfgang *et al.* (2000). The proteins are named using *P. aeruginosa* nomenclature. The steps that occur after cleavage of the pre-pilin by PilD are unknown and the components that interact with the mature PilA in the periplasm must still be identified. The results presented by Crowther and colleagues (2004) indicate that PilC may transmit the energy generated by the ATPases PilB and PilT to mediate pilus extension and retraction.

1. 9. 1. Colonization

Type IV pili from a variety of bacterial species are involved in colonization of both biotic and abiotic surfaces. Adherence to epithelial cells is mediated by the type IV pili of *P. aeruginosa* (Woods *et al.*, 1980), *N. gonnorhoeae* (Swanson, 1973), EPEC (Donnenberg *et al.*, 1992), *V. cholerae*, and *Salmonella enterica* serovar Typhi (Tsui *et al.*, 2003). Although the type IV pilins from these species are structurally alike and assemble into similar fibers, their binding specificities for host cell ligands differ.

Recent studies suggest that the Bfp of EPEC bind to LacNAc-like receptors on host cells as N-acetyllactosamine glycosides inhibit cellular adherence and bind to Bfp (Hyland *et al.*, 2006). Further research is necessary to determine if the major pilin BfpA mediates this interaction, or if an unknown minor pilin is responsible for cellular adherence. *N. gonorrhoeae* has a pilus tip protein, PilC, which mediates adherence to epithelial and endothelial cells (Nassif *et al.*, 1994; Rudel *et al.*, 1992; Rudel *et al.*, 1995b; Scheuerpflug *et al.*, 1999). This interaction is mediated by a complex motif composed of several linear amino acid sequences in the N-terminus of PilC (Morand *et al.*, 2001). The major subunit of the pilus, PilE, is also involved in adherence as variations in PilE mediate tissue tropism and *pilC* deficient pili hemagglutinate human erythrocytes (Rudel *et al.*, 1992; Scheuerpflug *et al.*, 1999). Previous studies suggested that CD46 was the specific PilC receptor on host cells (Kallstrom *et al.*, 1997). However, recent studies have demonstrated that CD46 is not the specific receptor, rather the specific receptor is a protein, and carbohydrates are not involved in this interaction (Kirchner and Meyer, 2005). The type IVb pili of *Salmonella enterica* serovar Typhi also bind to a protein or more specifically CFTR, although this interaction is mediated by the major pilin subunit PilS (Tsui *et al.*, 2003).

The type IV pili of *P. aeruginosa* specifically bind to asialo-GM_1 and GM_2 moieties on host cell membranes and there is no known tip-associated minor pilin (Lee *et al.*, 1994). Binding is mediated through interactions of the pilus with the carbohydrate sequence β-GalNAc(1-4)β-Gal found in these glycosphingolipids (Sheth *et al.*, 1994). Adherence of *P. aeruginosa* to host cells through the type IV pilus is a tip-associated event that is mediated by the C-terminal region, or D region of the pilin structural subunit (Irvin *et al.*, 1989; Lee *et al.*, 1994). Structural features in this domain important for binding are the disulfide bonded loop and the β-turns (Irvin *et al.*, 1989; Wong *et al.*, 1995). Immunization with purified peptides of the D region confers protection against subsequent challenge with homologous *P. aeruginosa* strains (Sheth *et al.*, 1995). Passive immunization with antibodies to the C-terminal disulfide bonded peptide of *V. cholerae* likewise confers protection against subsequent challenge (Sun *et al.*, 1997). In addition, it was demonstrated that this domain of TcpA is involved in cell to cell aggregation and colonization by *V. cholerae* (Kirn *et al.*, 2000).

Consequently, adherence of type IV pili to epithelial cells can occur through the major or minor pilin subunits to either carbohydrate or protein moieties on the surface of host cells. There are several host cell events that are initiated by adherence of type IV pili to their surfaces. Binding to host cells by the type IV pili of *P. aeruginosa*, *N. gonorrhoeae*, and *Salmonella enterica* serovar Typhi is the first step in the process of bacterial internalization (Chi *et al.*, 1991; Comolli *et al.*, 1999b; McLeod Griffiss *et al.*, 1999; Plotkowski *et al.*, 1994; Zhang *et al.*, 2000). It is not clear why *P. aeruginosa* and *N. gonorrhoeae* are internalized as these bacteria are not intracellular pathogens. Once in intimate contact with the host cell *Salmonella enterica* serovar Typhi express a type III secretion system required to inject effectors into the host cytoplasm that mediate bacterial internalization (Schlumberger and Hardt, 2005). In addition, type IV pili binding by the above species can initiate host responses such as, secretion of cytokines, mobilization of Ca^{2+}, and apoptosis (DiMango *et al.*, 1995; Jendrossek *et al.*, 2003; Kallstrom *et al.*, 1998; Kube *et al.*, 2001; Plant and Jonsson, 2006; Wang *et al.*, 2005). Therefore, once specifically bound to host cells type IV pili can elicit a number of physiological effects.

1. 9. 2. Natural transformation

Natural transformation is a bacterial process that involves the uptake of DNA from the environment, and results in lateral acquisition of novel genetic elements in a hereditable form (Dubnau, 1999). This process is utilized by both gram-negative and gram-positive bacteria and is hypothesized to have evolved from a mechanism to use DNA as a carbon source, or to repair damaged DNA (Dubnau, 1999; Finkel and Kolter, 2001; Solomon and Grossman, 1996). It was originally demonstrated that type IV pili are required for natural transformation in *N. gonorrhoeae* (Rudel *et al.*, 1995a; Seifert *et al.*, 1990; Sparling, 1966). It was later demonstrated that type IV pili are also required for natural transformation in several other gram-negative bacteria including *D. nodosus* (Kennan *et al.*, 2001a), *L. pneumophila* (Stone and Kwaik, 1999b), the cyanobacterium *Synechocystis* sp. PCC6803 (Yoshihara *et al.*, 2001), the extremely thermophilic bacterium *Thermus thermophilus* strain HB27 (Friedrich *et al.*, 2002a) and *P. stutzeri* (Graupner *et al.*, 2000b). It is not surprising that type IV pili are involved in natural transformation, as natural transformation in gram-positive bacteria uses a system that is similar to a type II secretion system (Dubnau, 1999). The exact role type IV pili play during natural transformation is unknown, however, it has been suggested that the pili bind DNA in the environment and retract to bring DNA to the cell surface to interact with other components in the system (Hobbs and Mattick, 1993). Indeed, there is some evidence in support of the above hypothesis, as non-specific DNA binding is associated with the production of type IV pili in *N. gonorrhoeae* (Aas *et al.*, 2002), and type IV pilus retraction deficient strains of *N. gonorrhoeae* (Wolfgang *et al.*, 1998a) and *P. stutzeri* lose the ability to perform natural transformation (Graupner *et al.*, 2001). Further research is required to determine the exact crucial function that type IV pili play during natural transformation.

1. 9. 3. Twitching motility

Twitching motility is a pilus dependent, flagella independent form of bacterial movement that occurs over damp surfaces by extension, binding, and then retraction of the type IV pili (Bradley, 1980; Mattick, 2002). Twitching motility can occur on a number of different surfaces including epithelial cells, metal, plastics, and glass, and therefore plays an important function during colonization of diverse substrates (Mattick, 2002). Recently, it was demonstrated that adherence of *P. aeruginosa* to inorganic substrates such as stainless steel and polyvinylchloride is also mediated by the C-terminal receptor binding domain of the PilA subunit, which is required for adherence to epithelial cells (Giltner *et al.*, 2006; Irvin *et al.*, 1989). Twitching motility is an important function of type IV pili and is involved in other bacterial processes such as colonization during infection (Bieber *et al.*, 1998; Comolli *et al.*, 1999a), natural transformation (Graupner *et al.*, 2001; Wolfgang *et al.*, 1998a), and biofilm formation (Chiang and Burrows, 2003a; O'Toole and Kolter, 1998). Twitching and social gliding motility have been studied extensively in several species including *N. gonorhoeae*, *P. aeruginosa*, and *M. xanthus*.

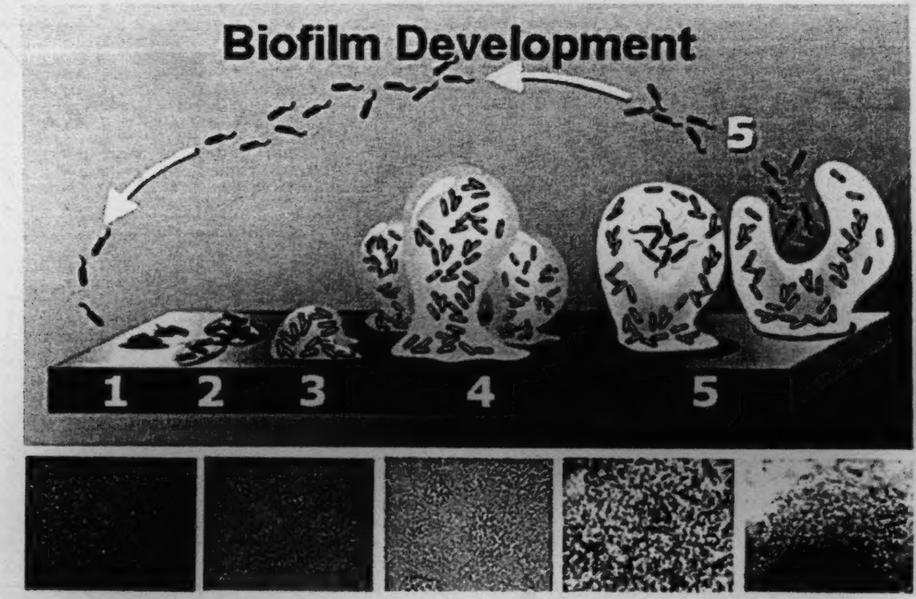
The original studies on twitching motility by Bradley (1972) demonstrated that the type IV pili of *P. aeruginosa* retracted into the cell and that adsorption of bacteriophages prevented this action. Real time pilus extension and retraction has been observed for *P. aeruginosa* at rates of ~0.5 μm s^{-1} (Skerker and Berg, 2001). In addition, studies using laser tweezers and *N. gonorrhoeae* demonstrated that pilus retraction can exceed forces of 80 pN (Merz *et al.*, 2000). Pilus retraction is mediated by the PilT and PilU proteins of *P. aeruginosa* and the PilT protein of *N. gonorrhoeae* (Whitchurch *et al.*, 1991; Whitchurch and Mattick, 1994; Wolfgang *et al.*, 1998a).

The systems involved in the control of twitching motility are complex, involving all genes necessary for pilus biogenesis, plus additional genes, many only partially described. Chemosensory phosphotransfer signal transduction systems, similar to those that control flagella rotation, direct twitching motility in *P. aeruginosa* (Whitchurch *et al.*, 2004) and social gliding in *M. xanthus* (Sun *et al.*, 2000). The classic flagellum systems have methyl-accepting chemotaxis proteins (MCPs) in the inner membrane, which respond to environmental signals (Bren and Eisenbach, 2000). The MCPs induce autophosphorylation of a histidine kinase (CheA), which then phosphorylates a response regulator (CheY), which changes the rotation of the flagella (Bren and Eisenbach, 2000). Therefore, in the *P. aeruginosa* twitching motility system the CheY like response regulator would interact with the pilus ATPases PilB, PilT, and PilU to cause extension or retraction of the pili (Whitchurch *et al.*, 2004). In *M. xanthus* the Frz system controls cell reversal frequencies where the type IV pili are disassembled at one pole and reassembled at the opposite pole, which results in directed motility (Mignot *et al.*, 2005). Therefore, the chemosensory systems modulate pilus retraction and directional movement through disassembly of the pili at one pole and reassembly at the other pole (Mignot *et al.*, 2005; Whitchurch *et al.*, 2004). Consequently, twitching motility is a complex process that is highly regulated and involved in the colonization of a number of surfaces in many gram-negative bacteria.

1. 9. 4. Biofilm formation

Biofilms can be defined as a single species or a mixed species community of bacteria that are encased in an extracellular polymeric substance matrix and attached to abiotic or biotic surfaces (O'Toole *et al.*, 2000). Bacteria found in biofilms differ drastically from their counterparts living freely in the planktonic form and therefore, the development of biofilms is a genetically regulated process (Costerton *et al.*, 1995b; Lazazzera, 2005). Biofilms have been extensively studied in several species of gram-negative and gram-positive bacteria, including *P. aeruginosa*, *V. cholerae*, *E. coli*, and *Staphylococcus* spp. (O'Toole *et al.*, 2000). One of the hallmarks of bacterial biofilms is high resistance to antimicrobials, which makes treatment of biofilm associated infections on implanted devices, or in chronic CF lung infections, extremely difficult (Costerton *et al.*, 1999; Stewart, 2002). Biofilm formation can be separated into several distinct steps starting with attachment to a surface, followed by microcolony formation through cell to cell interactions, maturation to produce highly structured bacterial pillars or mushrooms flanked by water channels, and then dispersal to colonize new environments (Figure 1-9) (O'Toole *et al.*, 2000; Sauer *et al.*, 2002a).

Initial attachment can be mediated by a number of bacterial adherence factors, but often is associated with the ability to produce type IV pili in gram-negative bacteria. The type IV pili of *P. aeruginosa* mediate binding to a number of abiotic surfaces in a specific manner (Giltner *et al.*, 2006). Although the TCP of *V. cholerae* are required for colonization of the intestine, the production of a second type IV pilus, the mannose-sensitive haemagglutinin (MSHA) mediates binding to some abiotic surfaces (Watnick *et al.*, 1999). Recently, it was determined that the Bfp from EPEC are also involved in biofilm formation on



* Type IV pili are potentially involved at these developmental stages

Figure 1-9. A schematic diagram of the separate stages that occur during biofilm formation. 1. Initial reversible attachment to a surface. 2. Irreversable attachment and development of distinct microcolonies. 3. Activation of quorum sensing and production of EPS. 4. Biofilm maturation to produce the characteristic mushroom or pillar like structures. 5. Dispersal from the mature biofilm to colonize new surfaces and establish a new biofilm. The bottom micrographs of *P. aeruginosa* depict each separate stage and * indicate potential stages where type IV pili are involved or required. Adapted from a review (Stoodley *et al.*, 2002).

abiotic substrates (Moreira *et al.*, 2006). However, binding to abiotic surfaces may occur through alternative adhesins, as type IV pili are not necessarily required for the initial attachment during biofilm formation (Klausen *et al.*, 2003a; Klausen *et al.*, 2003b; O'Toole and Kolter, 1998; Watnick and Kolter, 1999).

Even though type IV pili may not be required for the initial attachment to surfaces during biofilm formation, twitching motility plays an important role during biofilm development by *P. aeruginosa*. It was originally demonstrated that *P. aeruginosa* type IV pili mutants could not progress to the microcolony stage of development (O'Toole and Kolter, 1998). In addition, it was demonstrated that wild type *P. aeruginosa* cells move across the surface to form cell-cell aggregates (O'Toole and Kolter, 1998). It was later demonstrated that microcolonies develop initially through clonal growth (Klausen *et al.*, 2003a; Klausen *et al.*, 2003b). Twitching motility is then required for colonization of the entire surface, and formation of the mushroom cap structures on top of the mushroom stalks formed by non-motile *P. aeruginosa* (Klausen *et al.*, 2003a; Klausen *et al.*, 2003b). Twitching motility may also play a role in biofilm dispersal as *P. aeruginosa* twitching motility mutants form exaggerated pillar structures (Chiang and Burrows, 2003a).

Once microcolonies are formed, the genetic determinants of biofilm maturation are turned on, which is partially dependent on quorum-sensing in *P. aeruginosa* (Davies *et al.*, 1998). Protein profiles determined that the Las quorum sensing system is turned on after microcolonies are visible (Sauer *et al.*, 2002a). In contrast, the Rhl quorum sensing system is not activated until microcolonies are thicker than 10 μm and colonization of the entire surface had occurred (Sauer *et al.*, 2002a). However, microarray profiles demonstrated that there were relatively few differences between planktonic and mature biofilm *P. aeruginosa*, including no difference in expression of quorum sensing genes (Whiteley *et al.*, 2001). Other data indicate that quorum sensing is maximally expressed by *P. aeruginosa* close to the substratum, and before the mature biofilm stage (De Kievit *et al.*, 2001). Interestingly, GacA is involved in *P. aeruginosa* biofilm development, which also feeds into the quorum sensing regulon (Parkins *et al.*, 2001). Therefore, many of the genes required for acute infection and virulence control, are also required for biofilm formation and chronic infection by *P. aeruginosa*. This is somewhat perplexing as these phenotypes are regulated in an opposite fashion (Section 1. 5. and Figure 1-4). Therefore, further research in this area is required to sort out these discrepancies. Interestingly, in *V. cholerae* virulence expression and biofilm formation are repressed by quorum sensing (Hammer and Bassler, 2003).

Maturation is associated with the production of the extracellular polymeric substance that is made up of a complex mixture of polysaccharides, nucleic acids, and proteins (Sutherland, 2001). DNA is an important component of *P. aeruginosa* biofilms and is required for biofilm development (Whitchurch *et al.*, 2002). Although the origin of the DNA in *P. aeruginosa* biofilms is unknown it could arise from the release of extracellular vesicles filled with DNA, a widely observed phenomenon in many bacterial species, including *P. aeruginosa* (Kadurugamuwa and Beveridge, 1995). Recently, it was demonstrated that DNA is released by *P. aeruginosa* in a quorum sensing dependent manner (Allesen-Holm *et al.*, 2006). Furthermore, DNA is found predominantly in the stalks and interface between the stalk and cap area of the mushroom shaped multicellular *P. aeruginosa* biofilm structures (Allesen-Holm *et al.*, 2006).

Therefore, type IV pili play an important role in biofilm formation in a number of gram-negative species. In addition, a growing number of reports indicate that DNA and quorum sensing are important factors during the development of mature biofilms by *P. aeruginosa*.

1. 10. Objectives

The aim of this thesis is to evaluate novel binding specificities of *P. aeruginosa* type IV pili and to re-evaluate what properties of the C-terminal receptor binding domain are important for these binding activities. The original hypothesis was that type IV pili bind to DNA, as data suggested there is a band of positive charge displayed in a helical manner along the length of the assembled pilus (Keizer *et al.*, 2001). In addition, DNA was found to be a major component of the biofilm matrix of *P. aeruginosa*, and type IV pili are required for biofilm formation (O'Toole and Kolter, 1998; Whitchurch *et al.*, 2002). The hypothesized pilus-mediated DNA binding would be sequence independent and therefore it was necessary to develop a novel DNA binding assay. Once this assay was developed, the ability of the type IV pilus from *P. aeruginosa* to mediate DNA binding could be tested. In addition, the properties of the pilus that might contribute to DNA binding could be tested using pilus specific monoclonal antibodies and engineered monomeric pilin subunits (Doig *et al.*, 1990; Hazes *et al.*, 2000; Yu *et al.*, 1994). As the ability to bind DNA has biological implications for biofilm formation on abiotic and biotic surfaces, the effects of exogenous DNA on *P. aeruginosa* colonization could also be tested. Studies with monoclonal antibodies MAb PKL1 and MAb PK99H suggested that the C-terminal receptor binding domain is flexible, and binding activities could potentially be regulated by an allosteric mechanism (Lee *et al.*, 1994; Yu *et al.*, 1994). Candidates for such regulation include the released *P. aeruginosa* quorum-sensing acyl-homoserine lactones. Therefore, the ability of these molecules to increase or decrease the binding properties of purified *P. aeruginosa* pili could be tested. This work describes novel functions and regulation of *P. aeruginosa* type IV pili that will help define the mechanisms associated with biofilm formation and establishment of chronic CF pulmonary infections.

Chapter 2

DNA Binding: a Novel Function of *Pseudomonas aeruginosa* Type IV Pili

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C.L. Giltner, G.F. Audette, and D.W. Keizer helped with revision of this manuscript. C.M. Slupsky, G.F. Audette, and B.D. Sykes designed and produced Figure 1. C.M. Slupsky also produced the CATH data. D.W. Bautista constructed the truncated His-tagged PAK and K122-4 pilins used in this study.

2. 1. Introduction

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen that is a major cause of nosocomial infections and a leading cause of hospital-acquired pneumonia (Cunha, 2001). *P. aeruginosa* is also a major cause of severe infections in immunocompromised individuals including HIV (Manfredi *et al.*, 2000) and neutropenic cancer patients (Maschmeyer and Braveny, 2000). Furthermore, *P. aeruginosa* can cause bacteremia in burn victims (Pruitt *et al.*, 1998) and ulcerative keratitis of the cornea in users of extended-wear soft contact lenses (Fleiszig and Evans, 2002). Individuals suffering from cystic fibrosis (CF) are normally colonized with *P. aeruginosa* by adolescence, which causes a severe persistent respiratory infection that results in extensive lung damage (Brennan and Geddes, 2002). Treatment of *P. aeruginosa* infections requires antimicrobial therapy, which is challenging due in part to a high intrinsic resistance to many antibiotics and disinfectants (Normark and Normark, 2002). Therefore, *P. aeruginosa* infections result in considerable morbidity and mortality of immunocompromised individuals.

After aspiration or inhalation, the initial step in the establishment of a *P. aeruginosa* infection is the adherence to susceptible host cells *via* type IV pili (Woods *et al.*, 1980). *P. aeruginosa* type IV pili are assembled from 15 kDa pilin monomers, and adherence of *P. aeruginosa* to host cells through the type IV pilus is a tip-associated event that is mediated by the C-terminal region of the pilin structural subunit (Lee *et al.*, 1994). Type IV pili are also important colonization factors for several other gram-negative human pathogens including

Neisseria gonorrhoeae, *N. menigiditis* (Merz and So, 2000), enteropathogenic *Escherichia coli* (Giron *et al.*, 1991), enterotoxigenic *E. coli* (Giron *et al.*, 1994), *Vibrio cholerae* (Taylor *et al.*, 1987) and *Legionella pneumophila* (Stone and Abu Kwaik, 1998). Although type IV pili have a central role during infection, these fibrous structures are multifunctional and therefore involved in other bacterial processes. In addition to their role as colonization factors, the type IV pili of many species also play a role in twitching motility (Mattick, 2002), biofilm formation (O'Toole and Kolter, 1998), natural transformation (Dubnau, 1999) and bacteriophage infection (Bradley, 1974; Bradley and Pitt, 1974).

Natural transformation is a process unique to bacteria and involves active DNA uptake from the environment that can result in the lateral (horizontal) acquisition of new genetic information into a heritable form (Lorenz and Wackernagel, 1994). The involvement of type IV pili in natural transformation has been noted for several gram-negative bacteria including *Thermus thermophilus* (Friedrich *et al.*, 2002b), *L. pneumophila* (Stone and Kwaik, 1999a), *N. gonorrhoeae* (Seifert *et al.*, 1990), and *P. stutzeri* (Graupner *et al.*, 2000a). Although *P. aeruginosa* produce type IV pili no evidence of natural transformation has been observed to date (Carlson *et al.*, 1983). However, the *pilA* gene of *P. aeruginosa* can restore natural transformation in a *P. stutzeri pilA* mutant (Graupner *et al.*, 2001) and complement a *pilE* mutant in *N. gonorrhoeae* (Aas *et al.*, 2002). Therefore, while *P. aeruginosa* itself is not transformation competent, the type IV pili from *P. aeruginosa* can function during the process of DNA uptake. Intriguingly, there is a link between the expression of type IV pili and the ability to bind DNA to the bacterial surface. Mutations that affect the expression of type IV pili in *N. gonorrhoeae* reduce the amount of non-specific DNA binding at the cell surface (Aas *et al.*, 2002). Furthermore, pilus-retraction deficient mutants of *P. stutzeri* bind more DNA than wild type bacteria, although the ability to uptake DNA is greatly reduced (Graupner *et al.*, 2001).

The above observations combined with the surface electrostatic profiles and structural data of the type IV pili from *P. aeruginosa* (Figure 2-1) led to the hypothesis that type IV pili may bind DNA non-specifically. Although this phenomenon may not be important for productive natural transformation, it may facilitate other pilus associated functions. We have thus investigated the ability of the type IV pili from *P. aeruginosa* to bind DNA. We demonstrate that PAK, K122-4, and KB-7 pili bind to DNA obtained from several sources and that DNA binding is specific, concentration dependent, and saturable. Binding is dependent on the intact pilus structure and is also a tip-associated event that occurs through dominant interactions with the backbone with a preference for pyrimidine bases.

2. 2. Material and Methods

2. 2. 1. Bacterial strains, DNA sources, and antibodies

PAK pili were produced from Pseudomonas aeruginosa *strain* PAK/2Pfs, a multi-piliated mutant of *P. aeruginosa* PAK (Bradley, 1974). K122-4 and KB-7 pili were expressed and purified from P. aeruginosa strain DB2, a pilus-deficient

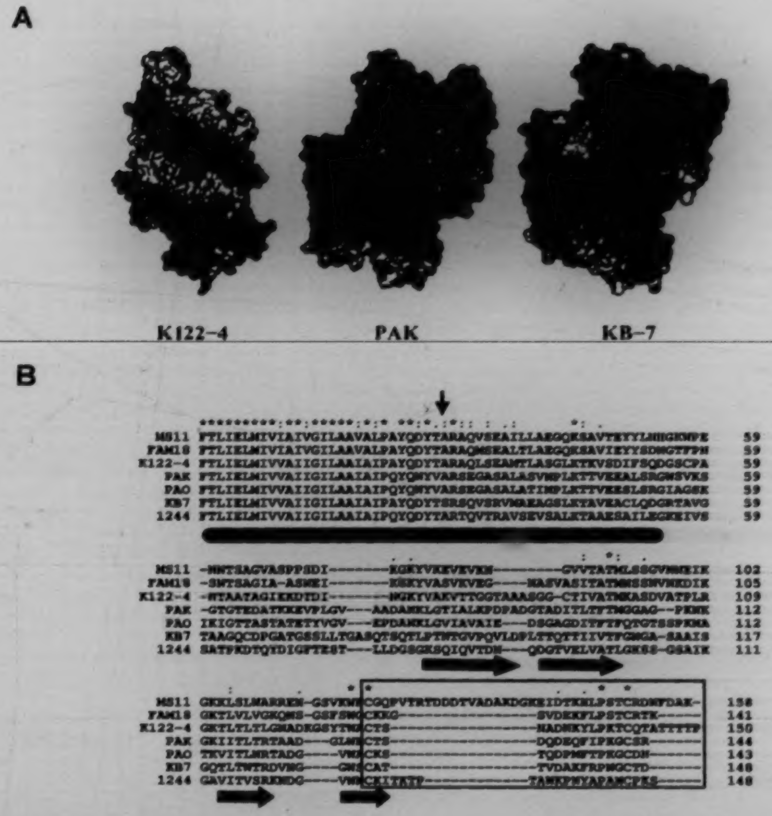


Figure 2-1. Amino acid sequence and structural aspects of *P. aeruginosa* type IV pilins. (A). Electrostatic surface representation generated using Delphi in InsightII of modeled pilins assembled into a pilus fiber (as described in reference 27) from *P. aeruginosa* strains K122-4, PAK and KB7. **Blue** represents positive, **red** negative, and **white** neutral charge. (B). Multiple sequence alignment of type IV pilins. * Indicated a strictly conserved residue, : indicates a highly conserved residue, and . indicates a moderately conserved residue and conserved secondary structural elements, based on an average of solved pilin structures, are indicated below each sequence block. The N-terminal α-helix is illustrated as a cylinder, and β-sheets are indicated with arrows. Production of soluble pilin monomers for structural studies is achieved by truncation of the 28 N-terminal residues, indicated by a vertical arrow (Hazes *et al.*, 2000). The disulfide-bound receptor binding loop is enclosed by a box. The listed sequences are from *N. gonorrhoeae* strain MS11 (gi|3212472), *N. meningitidis* strain FAM18 (gi|2228578), and *P. aeruginosa* strains K122-4 (gi|77636), PAK (gi|120438), PAO (gi|120440), KB7 (gi|3219798) and 1244 (gi|77632). The alignment was prepared with CLUSTALW with minor manual editing.

and retraction defective strain (Saiman *et al.*, 1990). *P. aeruginosa* cells were cultured in Luria-Bertani broth (L-B) or Tryptic Soy broth (TSB, Difco) at 37°C. *Escherichia coli* DH5α cells were used as the host cells for the purification of pUCP19 (Schweizer, 1991). E. coli DH5α harboring pUCP19 were cultured in L-B supplemented with 100 µg/mL carbenicillin (Sigma) and pUCP19 was purified using the QIAfilter™ Giga Kit (Qiagen). Lyophilized salmon sperm DNA (Roche), poly-phosphate (Sigma), and pyrophosphate (Fisher) solutions were prepared in 0.01 M phosphate buffer (PB) pH 7.4. Generation of the antibodies used in this study has been described previously: polyclonal PAK (Sastry *et al.*, 1985b), MAb PK3B, and MAb PK99H (Doig *et al.*, 1990).

2. 2. 2. Pili purification, biotinylation, and confirmation of activity

Purification of PAK pili, K122-4 pili, and KB-7 pili proceeded as described previously by Paranchych *et al.* (1979). Purification of pili was confirmed by analysis on 15% (w/v) SDS-PAGE according to standard procedures (Sambrook *et al.*, 1989), and electron microscopy (EM) after staining with 1% aqueous molybate pH 7.0 using a Hitachi H-700i EM operating at an accelerating voltage of 75 kV (Figure 2-2). His-K122-4 pilin and His-PAK pilin were constructed by inserting a His (5X) on the N-terminus of Δ(1-28) truncated pilins expressed off a PET expression vector in *E. coli* BL21. His-tagged pilins were purified from *E. coli* BL21 after induction with IPTG as follows. Cells were harvested by centrifugation and lysed by French press. Filtered lysate was applied to HiTrap™ Chelating HP (Amersham) Nickel column and imidazole eluted fraction

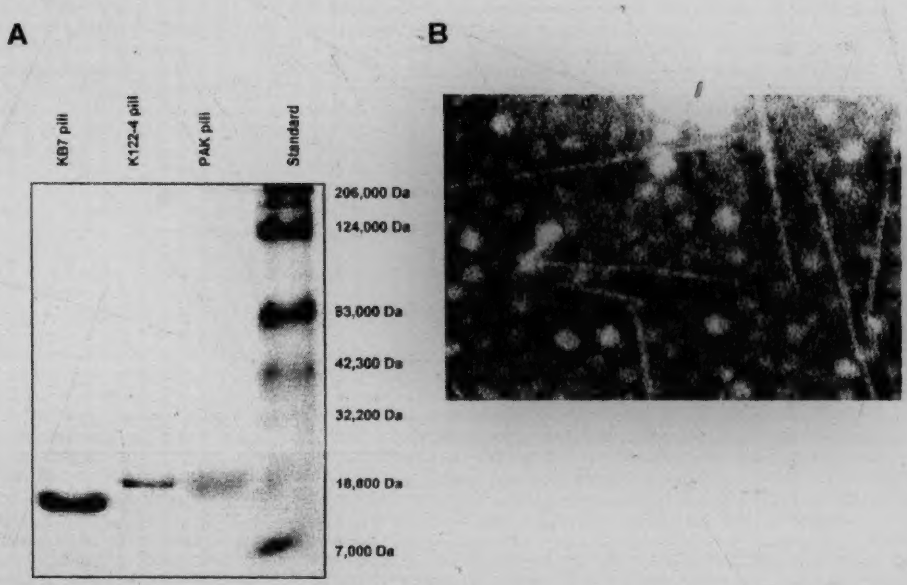

A
B
KB7 pili
K122-4 pili
PAK pili
Standard
206,000 Da
124,000 Da
83,000 Da
42,300 Da
32,200 Da
18,800 Da
7,000 Da

Figure 2-2. Purification of type IV pili from *P. aeruginosa* DB2 expressing KB7 pili, DB2 expressing K122-4 pili, and PAK2Pfs. (A). Purified KB7, K122-4, and PAK pili were analyzed on 15% (w/v) SDS-PAGE by standard procedures and then stained with coomassie blue. The pilus subunits are ~15kDa and all pilin subunits KB7, K122-4, and PAK were ~15kDa as expected (B). Electron microscopy (EM) of PAK pili after staining with 1% aqueous molybate pH 7.0 using a Hitachi H-700i EM operating at an accelerating voltage of 75 kV.

was then applied to a SP10/16 ion exchange column (Amersham) using an AKTA explorer HPLC (Amersham) with Unicorn version 3.00 software (Amersham).

The authenticity of the His-tagged truncated pilins was confirmed by N-terminal protein sequencing, mass spectroscopy, SDS-PAGE and by western blotting with appropriate antibodies. The procedure used for the biotinylation of the purified pili has been previously described (Yu *et al.*, 1996). The ability of the biotinylated pili to bind to asialo-GM_1 and GM_1 was determined to confirm the functional binding activity of the pili as previously described (Lee *et al.*, 1994).

2. 2. 3. Direct DNA binding assays

Poly-L-lysine (Sigma) was used to coat polystyrene microtitre plates (Corning) at a concentration of 20 µg/mL in 0.01 M sodium carbonate buffer pH 9.5 (100 µL per well) overnight at 4°C, followed by washing three times with phosphate buffered saline (0.01 M phosphate, 0.15 M NaCl (PBS) pH 7.4) containing 0.05% (w/v) bovine serum albumin (BSA) (Buffer A). The wells were then blocked with 5% (w/v) BSA in PBS, pH 7.4 overnight at 4°C and washed 3 times with buffer A. Salmon sperm DNA or pUCP19 was bound to the microtitre plates at a concentration of 60 µg/mL in 0.01 M phosphate buffer (PB) pH 7.4 (100 µL per well) overnight at 4°C and washed 3 times with buffer A. Biotinylated pili or His-tagged pilin were diluted in PB pH 7.4 containing 5 mM $MgCl_2$ and aliquoted 100 µL per well into the micotitre plates. The microtitre plates were then incubated for 1.5 hours at room temperature (RT) and washed 5 times with Buffer A.

Streptavidin(SA)-peroxidase (Sigma) or poly-His antibody (Sigma) were diluted 1:30,000 or 1:4000, respectively, into Buffer A and 100 µL per well was added to the plates. The plates were incubated for 1.5 hours at RT and then washed 5 times with Buffer A. Secondary rabbit anti-mouse-HRP (BioRad) diluted 1:3000 was added to plates containing His-tagged pilin and incubated for 1.5 hours and washed 5 times with Buffer A. Substrate buffer (0.01 M sodium citrate buffer pH 4.2 containing 1 mM 2,2'-Azino-di-[3-ethylbenzthiazoline-6-sulfonic] acid diammonium salt (ABTS) and 0.03% (v/v) hydrogen peroxide) was added 125 µL per well to each plate. The microtitre plates were incubated at RT for 10-45 minutes with shaking at 150 rpm. The absorbance was determined at 405 nm using a Multiskan Plus version 2.01 plate reader.

2. 2. 4. Competitive DNA binding assays

The microtitre plates were prepared as described above. A fixed concentration of biotinylated PAK (0.3 µg/mL), K122-4 (1.4 µg/mL), or KB-7 (3.5 µg/mL) pili were mixed with varying concentrations of pUCP19, salmon sperm DNA, poly-phosphate, or pyrophosphate. The reaction mixtures were incubated at RT for 30 minutes. Each reaction was aliquoted 100 µL per well into micotitre plates containing immobilized pUCP19 or salmon sperm DNA. The incubation times, wash procedures, and development of the plates were identical to those used in the direct DNA binding assays.

2. 2. 5. Direct and competitive oligonucleotide binding assays

The oligonucleotides used in this study are listed in Table 1. Double-stranded oligonucleotides were prepared by heating to 65°C and cooling to room temperature. PAK, K122-4, and KB-7 pili were used to coat polystyrene microtitre plates (Corning) at a concentration of 100 μg/mL in 0.01 M sodium carbonate buffer pH 9.5 (100 μL per well) overnight at 4°C, followed by washing 3 times with Buffer A. The wells were then blocked with 5% (w/v) BSA in PBS, pH 7.4 overnight at 4°C, followed by washing three times with Buffer A. The ds-biotinylated-oligonucleotide or mixtures of ss-biotinylated-oligonucleotide and ss-unlabeled-oligonucleotide were diluted into PB pH 7.4 containing 5 mM $MgCl_2$ and 100 μL aliquots were added to the wells of the microtitre plates and incubated at room temperature for 1.5 hours. The incubation times, wash procedures, and development of the plates were identical to those used in the direct DNA binding assays.

2. 2. 6. Antibody inhibition assays

Microtitre plates were prepared as described above. Biotinylated PAK pili were incubated with saturating amounts of antibody (MAb PK3B, MAb PK99H, or polyclonal PAK) for 30 minutes at RT. The reaction mixtures were then aliquoted 100 μL per well into the microtitre plates. The incubation times, wash procedures, and development of the plates were identical to those used in the direct DNA binding assays. Enzyme-linked immunosorbent assays (ELISA) were performed by coating microtitre plates with biotinylated or unlabeled PAK pili (100 μL per well) at 10 μg/mL in 0.01 M sodium carbonate buffer pH 9.5 overnight at

4°C. The plates were then washed 3 times with buffer A and blocked overnight with 5% (w/v) BSA in PBS, pH 7.4. Antibodies and SA-HRP were diluted in Buffer A and 100 µL aliquots per well were incubated in the microtitre plates for 1 hour at 37°C. Plates were washed 3 times with Buffer A and the secondary antibodies added. Either goat anti-rabbit-HRP or rabbit anti-mouse-HRP (BioRad) were used and incubated for 1 hour at 37°C. Plates were washed 3 times with Buffer A and 125 µL per well of substrate buffer was added followed by development and determination of the absorbance at 405 nm using a Multiskan Plus version 2.01 plate reader.

2. 3. Results

2. 3. 1. Pilin electrostatics and structural based hypothesis for a pilus-associated DNA binding function

An analysis of the pilus surface electrostatics of the left-handed modeled K122-4 pilus revealed a definite helical band of positive charge along the length of the fiber (Keizer *et al.*, 2001). Electrostatic surface analysis of right-handed pili modeled from the structure of PAK pilin (Hazes *et al.*, 2000) and the predicted structure of KB-7 pilin indicates that while the pilus surface electrostatics vary substantially among strains, a subtle helical pattern of solvent exposed positive charge is a feature of all three pili (Figure 2-1-A). Although these models are speculative, the electrostatic surface charges remain similar in other models of PAK pili (Craig *et al.*, 2004) and K122-4 pili (Audette *et al.*, 2004a; Audette *et al.*, 2004c; Keizer *et al.*, 2001). The pattern of positive charge along the length of the pilus is a conserved feature of the intact pilus that is evident even in the absence of sequence conservation among the various pilins (Figure 2-1-B). K122-4 pili have a more pronounced band of solvent exposed positive charge; this band is less distinct on the surface of PAK and KB-7 pili. Based on the electrostatics alone, K122-4 pili should have the highest affinity for DNA as K122-4 pili have the largest band of positive charge. PAK and KB-7 should have lower, but similar affinities for DNA as both pili have similar bands of positive charge.

Prediction of DNA binding through electrostatics is justified, since in general DNA-binding sites of proteins contain patches with large positive electrostatic scores (Jones *et al.*, 2003). Furthermore, examination of the β-sheet structure, which would produce the helical band of positive charge in the assembled pilus in the structures of the solved *P. aeruginosa* pilin monomers PAK (Hazes *et al.*, 2000) and K122-4 (Audette *et al.*, 2004a; Audette *et al.*, 2004c; Keizer *et al.*, 2001) reveals that this domain is dominated by solvent exposed Thr residues (Figure 2-1-B), which contribute to DNA binding in other proteins through interactions with the DNA backbone (Luscombe *et al.*, 2001). Therefore, we hypothesized that type IV pili of *P. aeruginosa* could mediate DNA binding through interactions with the DNA backbone and such binding should not display sequence specificity.

2. 3. 2. Pilus mediated DNA binding

Pili were purified as previously described (Paranchych *et al.*, 1979) from *P. aeruginosa* strain PAK/2Pfs, and from *P. aeruginosa* DB2 harboring K122-4 or KB-7 pilin expressed from the full length *pilA* from the respective strains cloned into pUCP19, and the purity of these pili was confirmed by SDS-PAGE and EM (Figure 2-2). Purified pili were biotinylated as previously described in order to probe pilus-DNA interactions (Yu *et al.*, 1996). As *P. aeruginosa* pili bind specifically to asialo-GM_1 (Lee *et al.*, 1994), binding of the biotinylated pili to asialo-GM_1 and GM_1 was examined to confirm that the biotinylated pili were functional (Figure 2-3). A novel assay was used to quantify DNA binding, as methods used to assay specific DNA binding like electrophoretic mobility shift assays (EMSA) would not be appropriate for the hypothesized non-specific DNA binding activity of *P. aeruginosa* pili. Therefore, pUCP19 or salmon sperm DNA was immobilized into the wells of microtitre plates through electrostatic interactions with immobilized poly-L-lysine.

Pili from all three strains of *P. aeruginosa* examined bound to immobilized DNA in a concentration dependent and saturable manner, albeit with different apparent affinities (Figure 2-4). As purified pili are variable in length it is not possible to calculate molarity. However, estimates of the binding affinity based on binding isotherms can be calculated. PAK exhibited the highest apparent affinity for DNA with a Kd of ~0.5 µg/mL followed by ~1.3 µg/mL for K122-4 and KB-7 had the lowest affinity at ~4.5 µg/mL (Figure 2-4). This corresponds to a 2.6-fold difference in apparent affinity between PAK and K122-4, a 9-fold difference between PAK and KB-7, and a 3.4-fold difference between K122-4

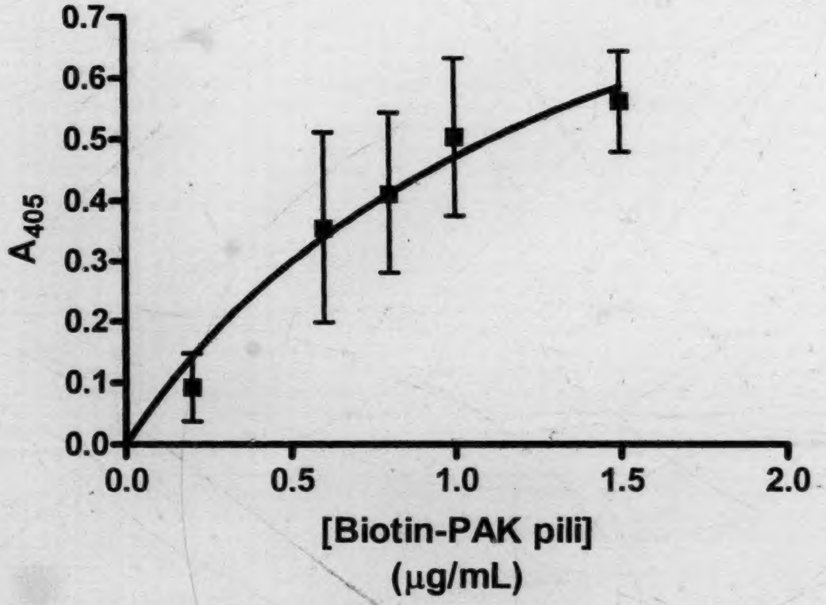


Figure 2-3. Direct binding of purified biotinylated PAK pili to asialo-GM_1. Asialo-GM_1 or GM_1 were immobilized into the wells of microtitre plates at a concentration of 0.5μg/mL in methanol by overnight evaporation at 4°C. The plates were then blocked with 3% BSA overnight. Biotinylated PAK pili were added to the plates at varying concentrations and incubated at 37°C for 1 hour. Binding was quantified spectrophotometrically using streptavidin-HRP. Binding to GM_1 is non-specific and was used as background for each representative biotinylated PAK concentration. The results presented are the means and standard deviations of at least 3 replicates from 2 independent experiments.

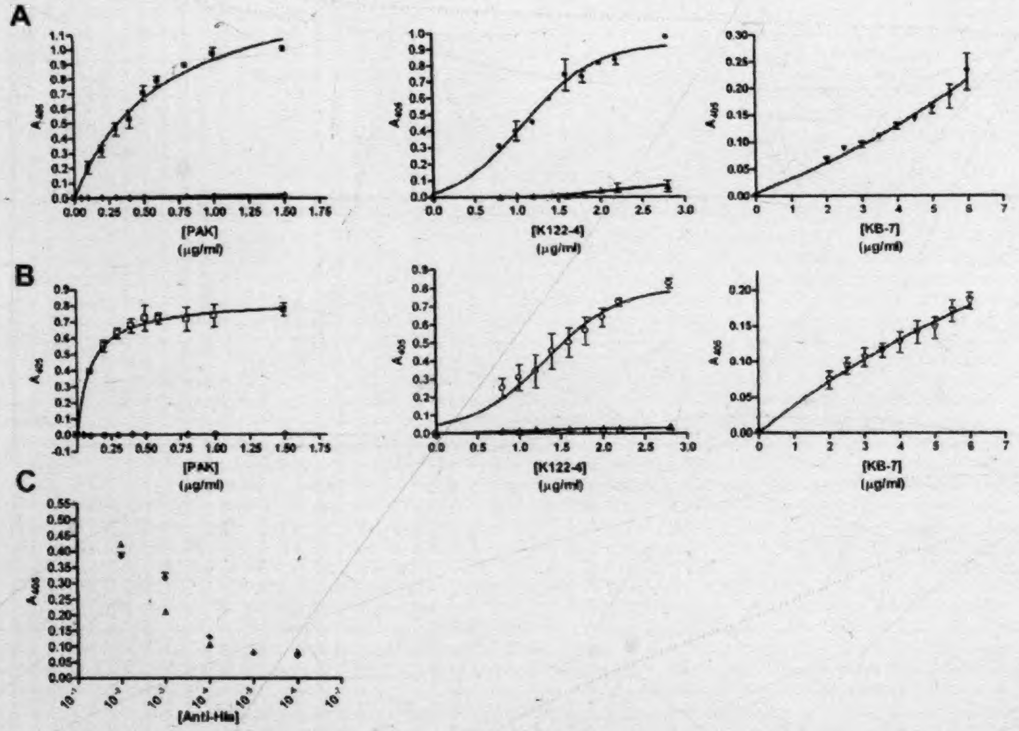


Figure 2-4. Concentration dependence of DNA binding by purified biotinylated pili from *P. aeruginosa* strains PAK, K122-4, and KB7. (A) pUCP19 plasmid (solid symbols) or (B) Salmon sperm DNA (open symbols) was immobilized into the wells of microtitre plates using poly-L-lysine. Varying concentrations of biotinylated PAK (■), K122-4 (•), KB7 (▼) pili or His-tagged PAK (◆) or K122-4 (▲) pilin were added to the plates and incubated for 1.5 hours at room temperature. Binding was quantified spectrophotometrically using streptavidin-HRP or primary mouse His antibodies and secondary HRP conjugate. The results presented are means and standard deviations of at least 3 replicates from 2 independent experiments. (C). ELISA assay of His-tagged PAK (◆) or K122-4 (▲) monomers. His-tagged monomers were immobilized into the wells of microtitre plates at 10 µg/mL. Antibody dilutions were added to the wells containing the immobilized His-tagged monomers in the presence of 5mM $MgCl_2$ and incubated for 1 hour at 37°C. Binding was quantified spectrophotometrically using anti-mouse-HRP and the results represent the means and standard deviations of 3 replicates.

and KB-7. Due to a low binding affinity, KB-7 binding to immobilized DNA did not saturate under the experimental conditions described, but saturation of KB-7 binding to DNA was observed when considerably higher KB-7 pili concentrations were employed (data not shown). These results were in opposition to the original hypothesis based solely on electrostatics that K122-4 should have the highest apparent affinity for DNA (Figure 2-1-A). Furthermore, as pili bound to both pUCP19 plasmid (Figure 2-4-A) and salmon sperm DNA (Figure 2-4-B), pili can bind both eukaryotic and prokaryotic DNA. Again it is not possible to determine if major differences exist between affinities for each type of DNA as the amount of DNA immobilized onto the plate can not be calculated, in addition to the inability to calculate the molarity of pili. The binding isotherm for K122-4 pili is distinctly sigmoidal in nature and may indicate cooperative binding. His-tagged monomers from K122-4 and PAK were used to evaluate whether binding is a function of the intact pilus. An ELISA assay determined that the poly-His antibody recognized our constructs (Figure 2-4-C) under conditions used in the binding assays. Monomers from PAK and K122-4 lacked the ability to bind DNA from both sources (Figure 2-4-A and 2-4-B) indicating that DNA binding is a function of the intact pilus.

2. 3. 3. Pilus mediated DNA binding is a specific event that involves interactions with the DNA backbone

To demonstrate that poly-L-lysine and biotinylation were not interfering with the pilus-mediated DNA binding, competitive binding assays were performed.

First, to demonstrate that poly-L-lysine has no effect on the ability of pili to bind DNA, purified biotinylated pili from the three strains were pre-incubated with either salmon sperm DNA or pUCP19 and then added to microtitre plates containing immobilized salmon sperm or pUCP19 DNA. Exogenous DNA from both sources reduced binding to immobilized DNA on the plate by more than 50% and in the case PAK pili over 90% inhibition was observed with both forms of DNA (Figure 2-5). The apparent affinity of both eukaryotic and prokaryotic DNA for pili is high, as the apparent Ki is < 5 nM (Figure 2-5). These observations suggest that DNA binding is specific, that both prokaryotic and eukaryotic DNA is recognized, and that the use of poly-L-lysine to immobilize DNA to the microtitre plates did not compromise the assay or generate a high degree of non-specific binding.

Unlabeled pili were able to inhibit DNA binding by biotinylated pili, indicating that biotinylation does not affect the ability of pili to bind to DNA (Figure 2-6). The structure-based hypothesis that DNA binding would be mediated by residues on the pilin exposed in the β-sheet with the phosphate backbone of the DNA was tested by employing poly-phosphate and pyrophosphate as competitors in binding assays with immobilized pUCP19 and salmon sperm DNA. Poly-phosphate but not pyrophosphate was able to inhibit binding to immobilized DNA by more than 50% and in the case of KB-7 pili by more than 90% (Figure 2-7-A and 2-7-B). These data suggest that the interaction between KB-7 pili and DNA is almost completely dependent on electrostatics. However, there is also a hydrophobic component to binding as increasing the salt concentration increases

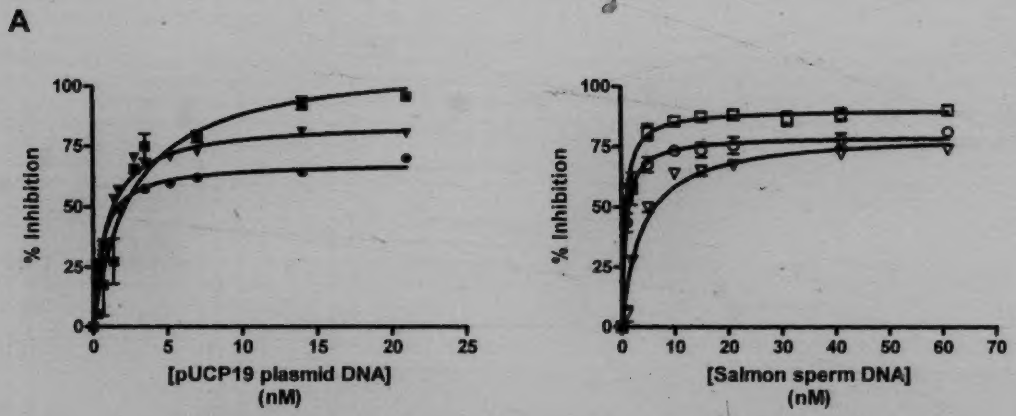


Figure 2-5. Exogenous DNA inhibits binding of biotinylated pili to immobilized DNA. pUCP19 plasmid (A). (closed symbols) or salmon sperm DNA (B). (open symbols) was immobilized into the wells of microtitre plates using poly-L-lysine. Biotinylated PAK (■), K122-4 (•), or KB7 (▼) pili were pre-incubated with varying amounts of exogenous DNA for 30 minutes. The reactions were then added to microtitre plates containing immobilized DNA and incubated for 1.5 hours at room temperature. Binding was quantified spectrophotometrically using streptavidin-HRP. The results presented are the means and standard deviations of at least 3 replicates from 2 independent experiments.

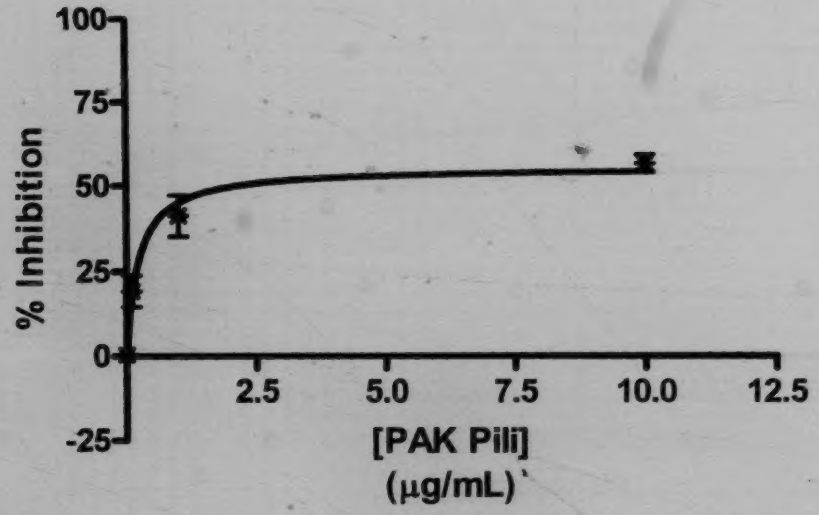


Figure 2-6. Unlabeled PAK pili inhibit binding of biotinylated PAK pili to immobilized DNA. Varying concentrations of unlabeled PAK pili were added to biotinylated PAK pili at a set concentration of 0.3 μg/mL. The reactions were then added to microtitre plates containing immobilized DNA and incubated for 1.5 hours at room temperature. Binding was quantified spectrophotometrically using streptavidin-HRP. The results presented are the means and standard deviations of at least 3 replicates from 2 independent experiments.

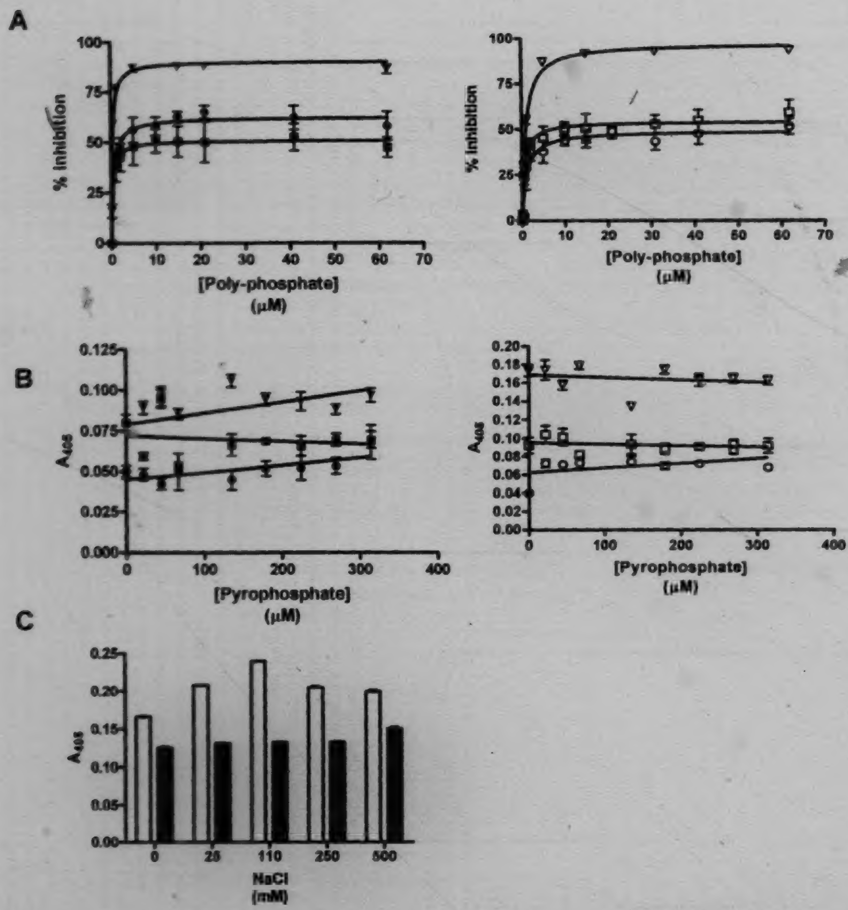


Figure 2-7. Polyphosphate and not pyrophosphate inhibits binding of biotinylated pili to immobilized DNA. pUCP19 plasmid (closed symbols) or salmon sperm DNA (open symbols) was immobilized into the wells of microtitre plates using poly-L-lysine. Biotinylated PAK (■), K122-4 (●) or KB7 (▼) pili were pre-incubated with varying concentrations of polyphosphate (A). or pyrophosphate (B). for 30 minutes. Reactions were then added to microtitre plates containing immobilized DNA and incubated for 1.5 hours at room temperature. Binding was quantified spectrophotometrically using streptavidin-HRP. The results presented are the means and standard deviations of at least 3 replicates from 2 independent experiments. (C). Salt dependence of pilus-mediated DNA binding. Biotinylated K122-4 (open bars) or KB-7 pili (black bars) were added to microtitre plates containing immobilized pUCP19 at 0.5 μg/mL in 0.01 M PB pH 7.4 containing varying amounts of NaCl and incubated for 1.5 hours at RT. Binding was quantified spectrophotometrically using streptavidin-HRP. The results presented are the means and standard deviations of 3 replicates.

the binding to immobilized DNA (Figure 2-7-C). K122-4 and PAK pili showed similar binding patterns in the presence of different salt concentrations (Figure 2-7-C and data not shown). These observations support the hypothesis that interactions with the DNA backbone are important for DNA binding by type IV pili, but suggest that there is also a specific component to DNA binding.

2. 3. 4. Pilus mediated DNA binding is not sequence specific although binding to pyrimidine bases is preferred

To evaluate the potential that DNA binding is also partially dependent on nucleotide sequence or base composition, short oligonucleotides were employed. A preferred DNA binding sequence was unknown, however it has been demonstrated that *pilA* effectively complemented a *pilE* mutant in *N. gonorrhoeae* (Aas *et al.*, 2002) and as the pUCP19 plasmid contains the sequence GCGGTGTGAA (which is almost identical to the *Neisseria gonorrhoeae* uptake sequence), a biotinylated-synthetic oligonucleotide containing the *N. gonorrhoeae* 10bp uptake sequence was employed.

The following complementary oligonucleotides were used to investigate this possibility; (the 10bp uptake sequence from *N. gonorrhoeae* is underlined): BIOTIN-ACTCGCCGTCTGAACCTA and TAGGTTCAGACGGCGAGT (Table 2-1). Purified PAK, K122-4, and KB7 pili were immobilized into the wells of microtitre plates and double-stranded biotinylated oligonucleotide was added at various concentrations. Binding to the ds-oligonucleotide was concentration dependent for PAK and K122-4, but no appreciable binding was observed for

Table 2-1. Oligonucleotides used for direct and competitive inhibition assays.

Oligonucleotide Name	Sequence
ss-NG	*ACTCGCCGTCTGAACCTA
ds-NG	*ACTCGCCGTCTGAACCTA TGAGCGGCAGACTTGGAT
BLAD	TGAGTATTCAACATTTCCGTGTCG
Poly-A	AAAAAAAAAAAAAAAAAA
Poly-T	TTTTTTTTTTTTTTTTTT
Poly-G	GGGGGGGGGGGGGGGGGG
Poly-C	CCCCCCCCCCCCCCCCCC

* Indicates location of biotinylation

KB7 pili (Figure 2-8-A). PAK and K122-4 pili bound the ds-oligonucleotide with roughly equivalent affinities, displaying Kds/apparent Kas of 57 nM / 1.8x10^7 M^{-1} and 50 nM / 2x10^7 M^{-1}, respectively (Fig. 2-8-A). All three types of pili were also able to bind to a ss-oligonucleotide containing the *N. gonorroheae* uptake sequence with slightly, but not significantly higher affinities compared to the ds-oligonucleotide (data not shown).

Competitive binding assays using immobilized PAK pili were then performed to determine if binding to the *N. gonorrhoeae* uptake sequence was specific. An unrelated ss-oligonucleotide of approximately the same length (BLAD; see Table 2-1) was used to inhibit binding of the ss-NG-oligonucleotide to the immobilized pili (Figure 2-8-B). The ability of this oligonucleotide to inhibit binding indicates that the pilus-mediated DNA binding is not specific for the *N. gonorrhoeae* uptake sequence. Again, binding of the ss-oligonucleotides to immobilized PAK pili is not influenced by the biotin-tag as S1 nuclease treated ss-biotin-oligonucleotide was unable to bind (Figure 2-8-C). To evaluate the possibility that base composition influenced oligonucleotide binding, I employed unlabeled single stranded poly A, T, G, and C oligonucleotides (Table 2-1) as solution phase competitors in binding assays with the single stranded biotinylated-*N. gonorrhoeae* uptake sequence. Poly-C and poly-T oligonucleotides were able to inhibit binding of the PAK pili to the *N. gonorrhoeae* uptake sequence (Figure 2-8-B). However, poly-A and poly-G oligonucleotides were unable to inhibit binding of the PAK pili to the *N. gonorrhoeae* uptake sequence (Figure 2-8-B). Therefore, pyrimidine base interactions may be favored over purine base

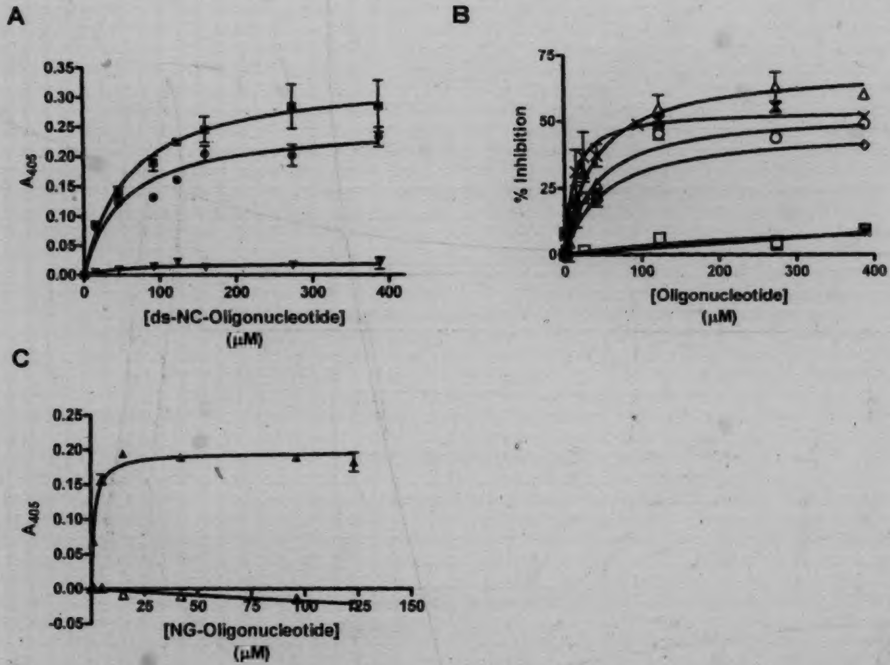


Figure 2-8. Direct and competitive binding assays using biotinylated oligonuceotides to immobilized *P. aeruginosa* pili purified from strains PAK, K122-4, and KB7. (A). Concentration dependence of binding a biotinylated double-stranded oligonucleotide containing the *N. gonorrheae* uptake sequence (Table 1) to immobilized PAK (■), K122-4 (•), or KB7 pili (▼). Varying concentrations of the ds-NG-oligonucleotide were added to the plates containing the immobilized pili and incubated for 1.5 hours at room temperature. Binding was quantified spectrophotometrically using streptavidin-HRP. The results presented are the means and standard deviations of at least 3 replicates from 2 independent experiments. (B). Direct competitive binding assay using single-stranded oligonucleotides (Table 1) against biotinylated ss-NG-oligonucleotide to immobilized PAK pili. 15nM of biotinylated ss-NG-oligonucleotide was mixed with varying concentration of unlabeled NG (Δ), BLAD (X), poly-C (○), poly-A (□), poly-T (◊), or poly-G (*) added to the microtitre plates containing immobilized PAK pili and incubated for 1.5 hours. Binding was quantified spectrophotometrically using streptavidin-HRP. The results presented are the means and standard deviations of at least 3 replicates from 2 independent experiments. (C). Direct binding of S1 nuclease treated ss-NG (Δ) or untreated ss-NG (▲) to immobilized PAK pili.

interactions during pilus-mediated DNA binding in the absence of apparent sequence specificity.

2. 3. 5. DNA binding is a tip-associated event that is dependent on the quaternary structure of the pilus

In order to determine which attributes of the intact pilus are involved in mediating DNA binding a series of antibody inhibition assays were performed. As I had established that DNA binding is dependent on the intact pilus structure as pilin monomers were unable to bind DNA (Fig. 2-4-A and 2-4-B), the relative importance of the pilus base and tip in DNA binding were examined. Monoclonal antibodies including MAb PK3B and MAb PK99H, which are specific for the base of the pilus and the C-terminal receptor binding domain involved in adherence to asialo-GM$_1$ (Doig *et al.*, 1990), respectively, and polyclonal PAK antibodies (Sastry *et al.*, 1985b) were employed in this study. PAK pili were pre-incubated with saturating amounts of antibodies and then added to microtitre plates containing immobilized pUCP19 plasmid. Pre-incubation with MAb PK3B produced only marginal inhibition compared to PAK pili probably through steric interference (Figure 2-9-A). Pre-incubation with both MAb PK99H and polyclonal PAK antibodies caused a large reduction in binding to the immobilized pUCP19 (Figure 2-9-A). These data indicate that DNA binding is a tip-associated event that requires other attributes of the intact pilus, since polyclonal PAK pili antibodies caused further reduction in binding compared to MAb PK99H. Biotinylation of PAK pili has no effect on antibody recognition nor does

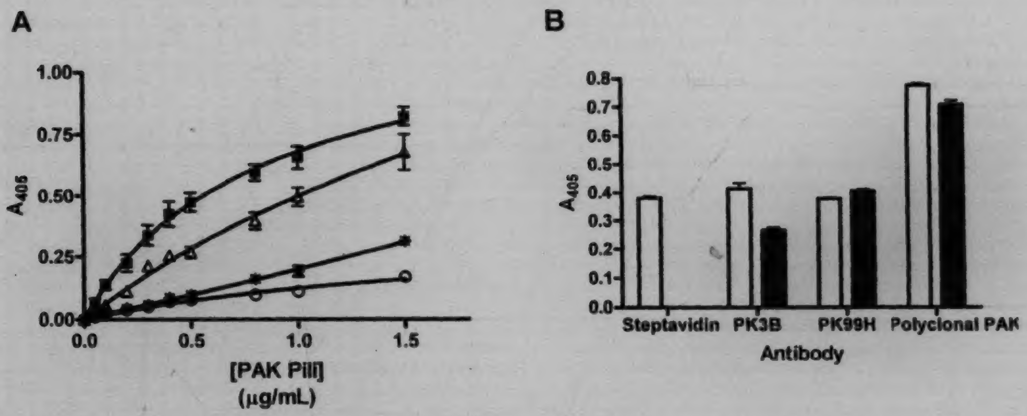


Figure 2-9. Antibody inhibition of binding biotinylated PAK pili to immobilized pUCP19. pUCP19 was immobilized into the wells of microtitre plates using poly-L-lysine. (A). Biotinylated PAK pili was either untreated (■) or pre-incubated with PK99H (*), PK3B (Δ), or polyclonal PAK (○) antibodies for 45 minutes at room temperature. Reactions were then added to microtitre plates containing immobilized pUCP19 plasmid and incubated for 1.5 hours at room temperature. Binding was quantified spectrophotometrically using streptavidin-HRP. The results presented are the means and standard deviations of at least 3 replicates from 2 independent experiments. (B). ELISA using immobilized unlabeled (black bars) or biotinylated (white bars) PAK pili and streptavidin-HRP, PK99H, PK3B, and polyclonal PAK. Results presented are the means and standard deviation of 3 replicate wells.

streptavidin recognize unlabeled PAK pili (Figure 2-9-B). Since DNA binding was a tip-associated event but involved the intact pilus structure, the relative contribution of the C-terminal receptor binding domain to DNA binding was determined. A direct competition of biotinylated PAK pili and PAK(128-144)ox peptide (Lee *et al.*, 1994) was performed. The PAK(128-144)ox peptide, which is an analog of the C-terminal receptor binding domain (Lee *et al.*, 1994) was unable to inhibit pilus-mediated DNA binding even at concentrations 1000 fold higher than the biotinylated PAK pili (Figure 2-10). These observations again support the hypothesis that the intact pilus structure is required for DNA binding and although DNA binding is tip-associated the binding domain does not appear to overlap with the C-terminal binding domain involved in adherence to asialo-GM$_1$.

2. 4. Discussion

Electrostatic modeling of the solvent exposed surface of K122-4, PAK, and KB-7 modeled pili suggests that a band of positive charge may be a common feature of type IV pili (Fig. 2-1-A). This band is present in a groove formed by the β-sheet domain found in the pilin monomers (Audette *et al.*, 2004a; Hazes *et al.*, 2000; Paranchych *et al.*, 1979). Examination of this domain in PAK (Hazes *et al.*, 2000) and K122-4 (Audette *et al.*, 2004a) reveals that there are a large number of solvent exposed Thr residues in the region (Fig. 2-1-B), a residue that has

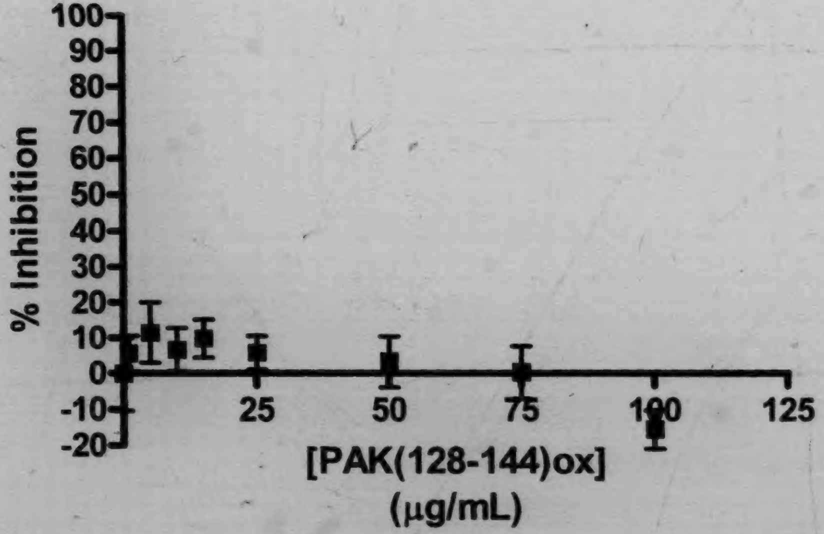


Figure 2-10. The synthetic peptide PAK(128-144)ox representing the C-terminal receptor binding domain does not inhibit binding of biotinylated PAK pili to immobilized DNA. Varying concentrations of PAK(128-144)ox were added to biotinylated PAK pili at a set concentration of 0.3 μg/mL. The reactions were then added to microtitre plates containing immobilized DNA and incubated for 1.5 hours at room temperature. Binding was quantified spectrophotometrically using streptavidin-HRP. The results presented are the means and standard deviations of at least 3 replicates from 2 independent experiments.

been found to mediate DNA backbone interactions in other DNA-binding proteins (Luscombe *et al.*, 2001). Furthermore, a number of solvent exposed Lys, Asn, and Gly residues were found in this area (Figure 2-1-B), and these side chains have also been previously ascribed roles in DNA binding events (Luscombe *et al.*, 2001). The CATH protein structural classification database (Orengo *et al.*, 1997) indicates that the pilin structure may be classified as a 2-layer sandwich of the α-β folding class. The closest structural match to pilin that contains only this architecture appears to be the major coat proteins of icosahedral RNA phage belonging to the Leviviridae family, which include bacteriophages MS2, Ga, Q-beta and PP7. These are simple viruses containing a single stranded positive-sense RNA molecule, a maturation protein, and 180 identical coat protein molecules that form the shell of the virus and bind the phage RNA forming an icosahedral lattice with a triangulation number of 3 (Golmohammadi *et al.*, 1993). MS2 coat protein dimers bind RNA through direct interaction of solvent exposed side chains of β-sheet residues, and where Ser and Thr side chains contribute directly to a purine binding specificity (Helgstrand *et al.*, 2002). Interestingly, there is a solvent exposed Thr residue at position 98 in the K122-4 structure, which is conserved in the β-sheet structure of all type IV pili sequences examined to date (Figure 2-1-B) (Audette *et al.*, 2004a). This domain has additional solvent exposed Thr residues (Figure 2-1-B) and these additional Thr residues may contribute to a pyrimidine binding preference. Thr98 may be conserved to facilitate DNA binding, as all variants of pili tested in this study maintain the ability to bind DNA even though the surface electrostatics vary.

I have shown that PAK, K122-4, and KB-7 pili were all able to bind directly to bacterial plasmid (Figure 2-4-A) and salmon sperm DNA (Figure 2-4-B) in a concentration dependent fashion. This suggests that the polymorphism in the pilin proteins (Hahn, 1997) does not affect the ability of the assembled pilus to bind to DNA. However, polymorphism in the pilin proteins does affect the affinity of the intact pilus for DNA as PAK had the highest apparent affinity followed by K122-4 and KB-7 pili respectively. In addition, DNA binding is a function of the intact pilus structure as pilin monomers are unable to bind to DNA from either source (Figure 2-4-A and 2-4-B). These observations indicate that the DNA binding site spans at least two pilin monomers or that the assembled monomer conformation in the pilus differs from the conformation of an unassembled monomer. Interestingly, Audette *et al.* (2004c) recently proposed that the K122-4 monomeric pilin may have a conformation in solution that differs from the pilin structure solved by X-ray crystallography (Audette *et al.*, 2004a). Although pilus-mediated DNA binding has been examined in *N. gonorrhoeae* and the conclusion drawn that pili do not bind DNA (Dorward and Garon, 1989; Mathis and Scocca, 1984), those studies were performed under conditions that would disassemble the pilus structure into monomeric units, and I have demonstrated that pilin monomers are unable to bind DNA (Figure 2-4-A and 2-4-B).

Non-specific DNA binding has previously been correlated to the expression of type IV pili composed of *P. aeruginosa* PAK pilin in *N. gonorrhoeae* (Aas *et al.*, 2002), and in multi-piliated retraction deficient mutants of *P. stutzeri* (Graupner *et al.*, 2001). Although this non-specific binding activity of the type IV pili may not be important for natural transformation, pilus-deficient strains of naturally transformable species have reduced DNA uptake potential (Friedrich *et al.*, 2002b; Graupner *et al.*, 2000a; Stone and Kwaik, 1999a). Furthermore, although *P. aeruginosa* is not naturally transformable, the type IV pili from this species can complement pilus mutants in natural transformation competent species (Aas *et al.*, 2002; Graupner *et al.*, 2000a), indicating that type IV pili have a conserved role during the process of natural transformation. Therefore, the non-specific DNA binding activity of type IV pili may increase the DNA uptake in naturally transformable species, potentially through retraction of the pilus to bring the DNA in contact with specific receptors on the cell surface (Fussenegger *et al.*, 1997). As functional type IV pili are not necessarily required for natural transformation (Graupner *et al.*, 2001; Long *et al.*, 2003), the non-specific DNA binding activity of the pili would only increase the amount of DNA available for uptake and therefore increase uptake frequency. In this regard, it is interesting to note that *P. aeruginosa* has homologues to several of the proteins required for DNA uptake in *N. gonorrhoeae* including ComE, ComA, and ComL (Stover *et al.*, 2000).

The different pili types displayed different apparent affinities for DNA. The results from both direct and competitive binding assays suggest that PAK pili have the highest apparent affinity for DNA followed by K122-4 and KB-7, respectively (Figures 2-4-A, 2-4-B, and 2-5). Pilus-DNA backbone interactions contribute significantly to pilus-mediated DNA binding as poly-phosphate is able to reduce the binding of K122-4 and PAK pili to both bacterial plasmid and salmon sperm DNA by more than 50% (Figure 2-7-A). Electrostatics and backbone interactions are dominant features of KB-7 pilus-mediated DNA binding as poly-phosphate could inhibit binding by more than 90% (Figure 2-7-A). The type IV pili of *P. aeruginosa* may potentially facilitate poly-phosphate uptake, as the results of this study indicate that pili can bind directly to poly-phosphate (Figure 2-7-A) and poly-phosphate active transport in *P. aeruginosa* is well documented (Poole and Hancock, 1984). After binding, pilus retraction could bring the poly-phosphate through the outer membrane to receptors on the inner membrane. Interestingly, poly-phosphate kinase and/or polyphosphate appear to be required for twitching motility (Rashid and Kornberg, 2000) and biofilm formation (Rashid *et al.*, 2000) in *P. aeruginosa*, two functions that also require type IV pili.

Although pilus-mediated DNA binding is dominated by electrostatics as DNA binding can be inhibited by poly-phosphate (Figure 2-7-A), other features of the DNA structure must also play a role in binding as the K122-4 pilus, which has the most positive β-sheet region (Figure 2-1-A) did not have the highest affinity for DNA, and binding studies performed in the presence of varying salt concentrations indicate that there is a specific component to DNA binding (Figure 2-7-C). Also, PAK and K122-4 pili, but not KB-7 pili were able to bind a ds-oligonucleotide with approximately the same apparent affinities (Figure 2-8-A). Therefore, short sequences of DNA are bound by PAK and K122-4 pili with similar energetics. Since KB-7 had a higher Kd for DNA (Figure 2-4) and because electrostatics dominate the interaction as poly-phosphate could inhibit

DNA binding by more than 90% (Figure 2-7-A) it is not surprising that a short oligonucleotide was unable to bind (Figure 2-8-A). In addition, it can be concluded that DNA binding is not dependent on a specific sequence as several oligonucleotides are able to bind PAK pili with similar apparent affinities (Figure 2-8-B). However, single stranded oligonucleotides containing only pyrimidine bases were able to inhibit binding of single stranded *N. gonorrhoeae* oligonucleotide indicating that pilus-mediated DNA binding displays base specificity or preference in the absence of sequence specificity (Figure 2-8-B). This apparent base composition preference may reflect either specificity of the DNA binding site on the pilus or specific physicochemical features of the single stranded oligonucleotides (i.e., the secondary structure of purine versus pryrimidine oligonucleotides) could influence binding.

DNA binding is a tip-associated event, but not a base-associated event with PAK pili as antibodies to the tip, but not the base of the pilus inhibited DNA binding (Figure 2-9-A). Additional features present in the quaternary structure of the pilus are also involved in DNA binding, as polyclonal PAK antibodies inhibited binding to a greater degree than the tip specific antibodies (Figure 2-9-A). This suggests that binding occurs both at or near the pilus tip and along the pilus filament. Furthermore, an analog of the C-terminal receptor binding domain of PAK pili (a synthetic peptide PAK(128-144)ox) and monomeric pilins are unable to inhibit pilus-mediated DNA binding (Figure 2-10) providing further support for the hypothesis that DNA binding is a function of the intact pilus.

Pilus-mediated DNA binding likely plays a role during biofilm formation as both extracellular DNA and type IV pili are required for *P. aeruginosa* biofilm formation (O'Toole and Kolter, 1998; Whitchurch *et al.*, 2002). DNA increases *P. aeruginosa* and type IV pilus binding to stainless steel which strongly suggests that the pilus DNA binding function plays a role in biofilm formation (van Schaik *et al.*, in preparation). DNA may aid in the formation of microcolonies as type IV pili are involved in the formation of cell clusters that require both cell-cell and cell-surface interactions (O'Toole and Kolter, 1998). Since pilus-mediated DNA binding is likely a multivalent interaction, aggregates of cells and DNA that are attached to a surface could potentially form. Interestingly, there is a high concentration of DNA in the lungs of patients with CF as a result of neutrophil necrosis (Tomkiewicz *et al.*, 1993) or specific secretion of DNA (Brinkmann *et al.*, 2004). This DNA may assist in the formation of a *P. aeruginosa* biofilm, and thus contribute to persistent colonization. Biofilm formation is involved in chronic colonization, as *P. aeruginosa* isolates from the lungs of CF patients have a mucoid phenotype characterized by the production of alginate, an exopolysaccharide produced during biofilm formation (Govan and Deretic, 1996). *P. aeruginosa* found in sputum obtained from CF patients are organized into clusters reminiscent of biofilms, produce quorum sensing agents required for biofilm formation, and are not associated with the mucosal epithelial surface (Singh *et al.*, 2000).

I have demonstrated that the type IV pili from three strains of *P. aeruginosa* can bind directly to multiple forms of DNA. This is a function of the intact pilus and potentially a tip-associated event involving other aspects of the quaternary structure. This binding event is dominated through interactions to the DNA backbone; however there is an apparent preferential interaction with the pyrimidine bases. The ability of the type IV pilus of *P. aeruginosa* to bind DNA could be involved in biofilm formation in the course of infection or during colonization of abiotic surfaces.

Chapter 3

Multiple Epitopes are Associated with the C-Terminal Receptor Binding Domain of *Pseudomonas aeruginosa* Type IV Pilus

A version of this chapter is in preparation for submission to the Journal of Bacteriology. van Schaik, E.J., Giltner, C.L., Hodges, R.S., and Irvin, R.T.

C.L. Giltner helped with revision of the manuscript. R.S. Hodges synthesized and conjugated the PAK pilin peptides used in this study.

3. 1. Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that is a leading cause of gram-negative nosocomial infections, which are difficult to treat because of a high resistance to antimicrobials (Obritsch *et al.*, 2005). *P. aeruginosa*, like most other gram-negative bacteria produce filamentous surface appendages termed pili or fimbriae. Type IV pili are one class of fimbriae that are associated with the ability to colonize the host, and in fact, pilus deficient strains of *Neisseria gonnorhoeae* (Swanson *et al.*, 1987), *Vibrio cholerae* (Herrington *et al.*, 1988), enteropathogenic *Escherichia coli* (Bieber *et al.*, 1998), and *P. aeruginosa* (Sato *et al.*, 1988; Tang *et al.*, 1995) are severely attenuated in their ability to cause infections. Although type IV pili play a central role during infection, these are multifunctional structures and are involved in several bacterial processes. The type IV pili of many species are also necessary for twitching motility (Mattick, 2002), biofilm formation (O'Toole *et al.*, 2000), natural transformation (Dubnau, 1999) and act as receptors for bacteriophage infection (Bradley, 1974).

The structural subunit of the type IV pilus is termed the pilin, of which there are two families, the type IVa and the type IVb (Craig *et al.*, 2004). The structures of several type IVa pilins from different species have been solved by X-ray crystallography (Audette *et al.*, 2004a; Hazes *et al.*, 2000; Parge *et al.*, 1995). These studies revealed a common structural architecture for type IVa pilins consisting of a long N-terminal α-helix packed onto a 4-stranded anti-parallel β-sheet (Craig *et al.*, 2004). In addition to the type IVa pilins, several of the type IVb pilin structures have been solved by X-ray crystallography (Craig *et* *al.*, 2003) and NMR (Ramboarina *et al.*, 2005; Xu *et al.*, 2004). The type IVb pilins are larger and have extra structural features, although the basic topology is similar to the type IVa pilins (Craig *et al.*, 2004). Both pilin types are assembled by a complex protein machine resembling the type II secretion system in gram-negative bacteria (Peabody *et al.*, 2003). The products of assembly are polar surface fibers that are approximately 6 nm wide and up to several micrometers long (Folkhard *et al.*, 1981).

Although the solved structures of several type IVa and type IVb pilins are available, the actual assembly and quaternary structure of the intact pilus remains a mystery. The current hypothetical models of the pilus have all been created assuming that the N-terminal α-helices form the central core of the pilus structure functioning as oligomerization domains. Therefore, in the intact pilus structure the N-terminal α-helices from each pilin subunit are found in coil-coiled bundles all the way through the interior of the pilus (Craig *et al.*, 2004). Most of the hypothetical type IVa pilus models are 1-start helix models that differ in the handedness and also the number of subunits per turn (Craig *et al.*, 2004; Hazes *et al.*, 2000; Keizer *et al.*, 2001; Parge *et al.*, 1995). In contrast, all the theoretical type IVb pilus models are 3-start left-handed helix models (Craig *et al.*, 2003; Ramboarina *et al.*, 2005; Xu *et al.*, 2004). Interestingly, a 3-start left-handed helix model has been proposed for PAK, a member of the type IVa pilus family (Craig *et al.*, 2004).

Additional information on the quaternary pilus structures has come from antibody studies, which have determined the surface exposed amino acid residues, and defined the binding domains. Polyclonal anti-pilus sera, for example has been used to identify surface exposed and immuno-dominant epitopes for *P. aeruginosa* strains PAK, PAO, and *N. gonorrhoeae* (Forest *et al.*, 1996; Sastry *et al.*, 1985b; Smart *et al.*, 1993). Although these studies have discovered similar surface exposed regions and differences in immuno-dominant epitopes, all agree that the C-terminal hypervariable disulfide bonded loop of both *P. aeruginosa* (Lee *et al.*, 1989a) and *N. gonorrhoeae* are immuno-dominant (Forest *et al.*, 1996). Further investigation demonstrated that this epitope is exposed both at the tip and along the length of the pilus from *N. gonorrhoeae* (Forest *et al.*, 1996), but evidence for the exposure of the C-terminal disulfide bonded loop in *P. aeruginosa* is contradictory. Lee *et al.* (1994) demonstrated that this domain is only exposed at the tip of the *P. aeruginosa* type IV pilus. Recent indirect evidence suggests that the C-terminal disulfide bonded loop is exposed along the entire length of *P. aeruginosa* pili (Smedley *et al.*, 2005). This latter study on the 1244 glycosylated pili revealed that the glycan attached to the C-terminal amino acid residue is exposed along the length of the pilus (Smedley *et al.*, 2005).

Antibody studies with *P. aeruginosa* have also demonstrated that the C-terminal disulfide bonded loop of the pilin is involved in cellular adherence (Doig *et al.*, 1990; Lee *et al.*, 1994). These studies were performed with two well characterized monoclonal antibodies (MAb) specific to the C-terminal receptor binding domain (RBD) of *P. aeruginosa* pili. The murine MAb PK99H recognizes amino acid residues 134-140 of the PAK pilin monomer (Wong *et al.*, 1992), inhibits binding to Buccal epithelial cells (Doig *et al.*, 1990), and is protective by passive immunization (Sheth *et al.*, 1995). The murine MAb PAK-13, however, is cross-reactive and recognizes the C-terminal RBD of several *P. aeruginosa* strains (Campbell *et al.*, 1997). In addition, passive administration of MAb PAK-13 provides protection against challenge with several *P. aeruginosa* strains (Sheth *et al.*, 1995). Both these monoclonal antibodies have been invaluable in determining the function of the C-terminal RBD, and in development of a synthetic peptide vaccine against *P. aeruginosa* infection (Cachia and Hodges, 2003). Another partially characterized murine MAb exists, MAb PKL1, recognizes the C-terminal RBD in direct ELISAs (Yu *et al.*, 1994) and confers protection against PAK *P. aeruginosa* challenge by passive immunization. The current study was undertaken to further characterize MAb PKL1 and explore the structure-function relationship of the C-terminal RBD in the native *P. aeruginosa* PAK pili.

MAb PKL1 bound with minimal affinity to the C-terminal peptides from PAK pilin with an endpoint titre of 10^{-4} corresponding to an antibody concentration of 2.3 µg/mL versus endpoint titres of 10^{-7} corresponding to an antibody concentration of 0.23 ng/mL for the intact PAK pilus and monomeric PAK pilin subunit. As MAb PKL1 has minimal affinity for the linear peptides of PAK pilin (including the C-terminal RBD) therefore MAb PKL1 must recognizes a 3-dimensional epitope present in the PAK pilin. In addition, the MAb PKL1 epitope does not overlap with the MAb PK99H antigenic epitope. I propose that the MAb PKL1 epitope is associated with the loop and the connecting β-strands adjacent to the C-terminal RBD. Interestingly, MAb PKL1 is significantly more effective than MAb PK99H at inhibiting epithelial cell binding. Furthermore, it was demonstrated that the C-terminal RBD is exposed along the length of the PAK pilus, which supports the 3-start left-handed helix model of the intact pilus described by Craig *et al.* (2004). These results have important implications for the binding functions of the C-terminal RBD of the type IV pilus from *P. aeruginosa*.

3. 2. Material and Methods

3. 2. 1. Bacterial strains

P. aeruginosa strain K (Bradley and Pitt, 1974) and mutants of this parental strain including ΔPil, a strain constructed by deleting a region encoding most of the *pilA* and *pilB* genes (kindly provided by J. Boyd), and 2Pfs, a multi-piliated mutant (Bradley, 1974), were used in this study. *P. aeruginosa* strains were routinely grown in Luria-Bertani or tryptic soy broth at 37°C. PAK pili were purified from PAK 2Pfs as previously described (Paranchych *et al.*, 1979). The purity and structural integrity of the pili were confirmed by 15 % SDS-PAGE using standard methods (Sambrook *et al.*, 1989) and by electron microscopy using a Hitachi H-800i electron microscope operating at an accelerating voltage of 75 kV following staining with 1% aqueous molybate (pH 7.0). PAK pilin was purified using the previously constructed Δ(1-28) truncated PAK pilin expressed in *Escherichia coli* BL21, as described elsewhere (Hazes *et al.*, 2000). Purified

PAK pili and PAK bacteria were biotinylated using previously described protocols (Yu *et al.*, 1996). The protein concentrations of purified PAK pili, PAK pilin, MAb PK99H, and MAb PKL1 were determined using a BCA assay (Pierce). The functionality of the biotinylated PAK pili was determined by assessing the adherence to asialo-GM_1 and GM_1 as described previously (Lee *et al.*, 1994). The viability of the PAK wild type and mutant bacteria were determined following biotinylation using viable counts after serial dilution in phosphate buffered saline pH 7.4 (0.01 M phosphate pH 7.4, 0.15 M NaCl) (PBS).

3. 2. 2. Peptide synthesis and conjugation to protein carriers

The peptides used in this study were synthesized using the Merrifield solid-phase synthesis procedure (Gisin, 1973) on a peptide synthesizer (model 990 Beckman Instruments). The addition of protected amino acids and cleavage of the peptides from the resins has been described previously (Parker and Hodges, 1985). The cleaved peptides were then purified using HPLC on a SynChropak C18 column (Synchrom) by a previously described protocol (Lee *et al.*, 1989b). Where necessary, the intrachain disulfide bridges in the peptides were formed by air oxidation (Hodges *et al.*, 1988). The formation of the disulfide bridges was monitored using circular dichroism as previously described (Matsoukas *et al.*, 1984). The peptides were either used directly or conjugated to keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) via a photoactivated cross-linker, which was added during synthesis, while the peptides were still on the solid matrix (Parker and Hodges, 1985). The covalent attachment of the peptide carrier was performed by a previously described protocol (Lee *et al.*, 1989b). See Table 3-1 for the list of peptides used in this study and their amino acid sequences.

3. 2. 3. Enzyme linked immunoassays

ELISAs were performed by coating PAK pili or PAK peptide-BSA conjugates, at concentrations of 10 μg/mL and 5 μg/mL respectively in 0.01 M sodium carbonate buffer pH 9.5, into 96 well polystyrene plates (Corning) using standard procedures (Engvall, 1980). The plates were then blocked with 5 % (w/v) BSA in PBS, pH 7.4 overnight at 4°C. The plates were washed 3 times with 0.05 % (w/v) BSA in PBS, pH7.4 (buffer A) and antibody dilutions were added to the plates in buffer A. The monoclonal antibodies (MAb PK99H and MAb PKL1) used in this study have previously been fully (Doig *et al.*, 1990; Wong *et al.*, 1992) or in part characterized (Yu *et al.*, 1994) respectively. The polyclonal anti-PAK(128-144)ox serum used in this study as a positive control has been described previously (Lee *et al.*, 1989b). Competitive ELISAs were performed by pre-incubation of the antibodies with peptides, PAK pili, or PAK pilin for 30 minutes at 37°C.

Sandwich ELISAs were performed by coating 5 μg/mL or 10 μg/mL of MAb PKL1 and MAb PK99H respectively in 0.01 M sodium carbonate buffer, pH 9.5 into 96 well polystyrene plates (Corning). The plates were washed three times with buffer A and then blocked with 5% (w/v) BSA in PBS, pH 7.4 overnight at 4°C. Biotinylated PAK pili at a concentration of 2.5 μg/mL were pre-incubated with 10^{-2} to 10^{-6} dilutions of MAb PKL1 or MAb PK99H for 30 minutes at room

Table 3-1. The synthetic PAK pilin peptides of the C-terminal RBD used either un-conjugated or conjugated to KLH or BSA.

Peptide	**Sequence**
PAK(128-144)**ox**	Ac-K**C**TSDQDEQFIPKG**C**SK-OH
PAK(128-144)**red**	Ac-K**C**TSDQDEQFIPKG**C**SK-OH
PAK(116-144)**ox**	Ac-ITLTRTAADGLWK**C**TSDQDEQFIPKG**C**SK-OH
PAK(116-144)**red**	Ac-ITLTRTAADGLWK**C**TSDQDEQFIPKG**C**SK-OH
PAK(134-140)	Ac-DEQFIPK-amide
PAK(117-125)	Ac-TLTRTAADG-OH
PAK(137-144)	Ac-FIPKGCSK-OH

These peptides were synthesized by solid phase and are N-α-acetylated with a free carboxyl with the exception of PAK(134-140), which was synthesized as the N-α-acetylated amide peptide because of its short length. The peptides that had a formed disulfide bridge between cysteines 129 and 142 are labeled **ox**, while the un-oxidized forms are labeled **red**. The PAK(128-144)ox represents the native form of the peptide in the C-terminal RBD or the pilin.

temperature (RT) before being applied to the plates containing immobilized antibodies. Whole cell ELISAs were performed by coating *P. aeruginosa* at an OD_{600} at 1.0 diluted from an overnight culture in 0.01 M sodium carbonate buffer pH 9.5 into 96 well polystyrene plates (Corning). The plates were washed three times with buffer A and then blocked with 5% (w/v) BSA in PBS, pH 7.4 overnight at 4°C. The antibodies and biotinylated PAK cells or pili were detected by incubating mouse or rabbit 2nd antibody conjugates (Jackson Laboratories, Inc.) or streptavidin conjugated to horseradish-peroxidase (Sigma) for one hour at 37°C. The plates were then washed three times with buffer A followed by development by adding substrate buffer (0.01 M sodium citrate buffer pH 4.2 containing 1 mM 2,2'-Azino-di-[3-ethylbenzthiazoline-6-sulfonic] acid diammonium salt (ABTS) and 0.03% (v/v) hydrogen peroxide) for five to 20 minutes. The absorbance readings were measured at 405 nm using a Multiskan Plus version 2.01 plate reader.

3. 2. 4. Transmission immuno-electron microscopy

Purified PAK pili at a concentration of 1 µg/mL in PBS, pH 7.4 were applied to formvar-coated copper grids for ten minutes at RT. The grids were then blocked with 1% (w/v) skim milk (Fisher) and 0.1% (v/v) Tween 20 (Fisher) in PBS, pH 7.4. The grids were then washed two times with 0.1% (v/v) Tween 20 in PBS, pH 7.4 for three minutes. The rabbit polyclonal anti-PAK pili sera (Sastry *et al.*, 1985b) or the rabbit polyclonal anti-PAK(128-144)ox sera (Lee *et al.*, 1989b) were added to the grid at a 1:1000 dilution and incubated for one hour at RT.

The grids were washed three times with 0.1% (v/v) Tween 20 in PBS, pH 7.4 for three minutes. 20 nm Colloidal gold particles conjugated to goat anti-rabbit sera (E Y Laboratories) were added to the grids at a 1:100 dilution and incubated for one hour at RT. The grids were washed three times with 0.1% (v/v) Tween 20 in PBS, pH 7.4 for three minutes and two times in 0.22 µm filtered H_2O. The grids were then stained briefly with 1% phosphotungstic acid, pH 6 and the excess stain was removed with filter paper. Images were taken using a Hitachi H-700i electron microscope operating at an accelerating voltage of 75 kV.

3. 2. 5. Direct binding assays

The methods for assessing direct binding to asialo-GM_1 by purified PAK pili or *P. aeruginosa* cells have been described previously (Lee *et al.*, 1994). Briefly, glycosphingolipids in methanol were coated into 96 well polystyrene plates by evaporation overnight at 4°C. The plates were blocked with 3% (w/v) BSA at 37°C for one hour followed by three washes with buffer A. Direct binding to Buccal epithelial cells (BECs) was performed using a previously described protocol with some modifications (Sexton and Reen, 1992). 24 Well tissue culture plates (Corning) were coated with poly-L-lysine at a concentration of 50 µg/mL at 37°C for two hours followed by two washes with PBS, pH 6.8. BECs were collected from healthy volunteers using wooden applicator sticks into PBS, pH 6.8, and filtered through 70 µm nylon mesh. BECs were added to the plates at a concentration of 1 x 10^5 cells/mL as determined by direct counts using a hemacytometer and incubated at 37°C for 45 minutes. The plates were then centrifuged at 1000 x g for 10 minutes at 22°C. The unbound BECs were removed by aspiration and the plates were incubated overnight at 4°C with PBS, pH 6.8. The BEC plates were blocked with 2 mg/mL gelatin in PBS, pH 6.8 for two hours at RT and then washed three times with PBS, pH 6.8. The direct DNA binding assays were performed by a previously described protocol (van Schaik *et al.*, 2005). The above prepared plates were used for direct binding assays with biotinylated or unlabeled PAK pili or *P. aeruginosa* bacteria after a 30 minute pre-incubation with saturating concentrations of MAb PK99H or MAb PKL1. The plates were developed using the same method described above for ELISA assays.

3. 3. Results

3. 3. 1. Antibody titres to PAK pili and PAK pilin

The murine monoclonal antibody MAb PKL1 subtype IgG3(κ) has not been extensively characterized, but based on direct ELISAs using the synthetic peptide PAK(128-144)ox, appeared to have the same specificity as the murine monoclonal antibody MAb PK99H subtype IgG1(κ) (Doig *et al.*, 1990; Wong *et al.*, 1992; Yu *et al.*, 1994). The monoclonal antibody MAb PK99H recognizes an epitope comprising amino acids 134-140 of PAK pilin and is able to inhibit pilus mediated binding to host cell receptors (Doig *et al.*, 1990; Wong *et al.*, 1992). However, recent studies suggested that MAb PKL1 was not equivalent to MAb PK99H and thus I decided to further characterize MAb PKL1. Whole cell ELISAs determined that both MAb PKL1 and MAb PK99H recognized epitopes that were displayed on the exterior of *P. aeruginosa* PAK cells (Figure 3-1-A). Neither MAb PK99H nor MAb PKL1 had affinity for PAKΔPil (an isogenic derivative of the PAK wild type strain that does not produce pili) indicating that these antibodies do not recognize any non-specific epitopes displayed on PAK cells (Figure 3-1-A). In addition, both monoclonal antibodies were specific for PAK, which agrees with previous results (data not shown) (Doig *et al.*, 1990; Yu *et al.*, 1994).

The MAb PK99H and MAb PKL1 antibodies were titred against both PAK pili and the monomeric PAK pilin (Δ1-28), missing the first 28 residues and therefore the hydrophobic N-terminal α-helix (Figure 3-1-B) (Hazes *et al.*, 2000). MAb PKL1 and MAb PK99H had equivalent titres for PAK pili and monomeric PAK pilin (Figure 3-1-B). Even though the pilus is made up of approximately 1000 PAK pilin subunits, the ELISAs used equivalent protein concentrations. Therefore, although the PAK pilin subunits are present as a quaternary structure in the pilus, a similar number of PAK pilin subunits should be present under both conditions. Figure 3-1-B indicates that the epitope of the monoclonal antibody MAb PKL1 is not a quaternary epitope present only in the intact PAK pilus fiber, as the titres for the intact pilus and pilin subunit were similar. To obtain equivalent endpoint titres of 10^{-7}, protein concentrations of 0.23 ng/mL for MAb PKL1 and 0.4 ng/mL for MAb PK99H were required (Figure 3-1-B). All subsequent experiments were performed using concentrations of MAb PK99H and MAb PKL1 that result in equal titres for PAK pili. Interestingly, MAb PK99H had the same titre for both PAK pili and PAK pilin (Figure 3-1-B). This was

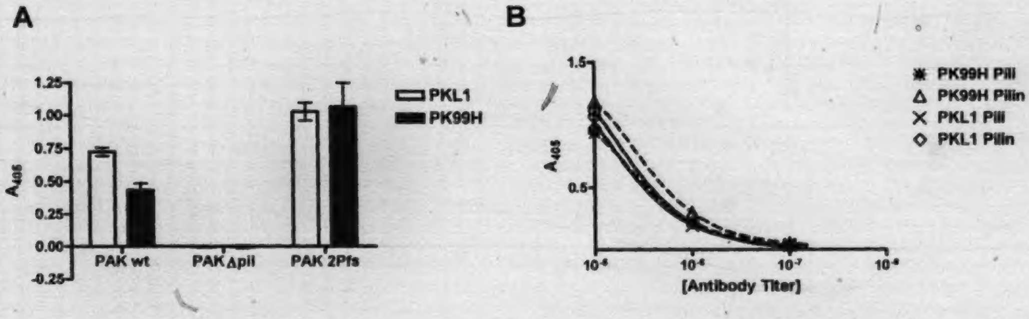


Figure 3-1. (A) Whole cell ELISA using *P. aeruginosa* strains PAK, a wild type strain, PAKΔPil, a pilus-deficient strain, and PAK2Pfs, a hyperpiliated strain. *P. aeruginosa* were coated into 96 well polystyrene plates at an OD_{600} of 1. The *P. aeruginosa* cells were detected using monoclonal antibodies PKL1 and PK99H. Development used rabbit anti-mouse 2° antibodies conjugated to horseradish peroxidase and ABTS. The absorbance readings were taken at 405nm. The results represent data from two independent experiments done in triplicate and error bars depict SEM. (B) Direct ELISA using purified PAK pili and monomeric PAK pilin (Δ1-28) missing the first 28 residues (Hazes *et al.*, 2000). Purified proteins were coated into 96 well polystyrene plates at a concentration of 5 µg/mL. Purified proteins were detected using PKL1 and PK99H. The development used rabbit anti-mouse 2° antibodies conjugated to horseradish peroxidase and ABTS. The absorbance readings were taken at 405nm. The results represent data from two independent experiments done in triplicate and error bars depict SEM.

unexpected as previous results suggest the MAb PK99H epitope is only displayed at the tip of the pilus (Lee *et al.*, 1994), in which case, it was expected that MAb PK99H would have a much high affinity for the PAK pilin. However, it is worth noting that the previous immuno-cytochemical studies used purified MAb PK99H IgG and indirect localization by colloidal gold-protein A. Potentially using this lower affinity probe to detect MAb PK99H was perhaps only effective at identifying clustered antibody binding sites (Lee *et al.*, 1994).

3. 3. 2. Antibody titres to PAK pilin peptides

PAK pilin peptides were used to determine the regions of the PAK pilin that each monoclonal antibody recognized and to determine if MAb PKL1 recognized a linear epitope present in the PAK pilin. As expected the monoclonal antibody MAb PK99H had affinity for peptides containing residues 134-140 or a combination of these residues from PAK pilin (data not shown) (Wong *et al.*, 1992). The endpoint titres of MAb PK99H for the synthetic peptide PAK(128-144)ox were the same as for the intact PAK pilus and the PAK pilin monomer (compare Figures 3-1-B and 3-2-B). Interestingly, MAb PKL1 had only marginal affinity for peptides containing the C-terminal RBD or amino acid residues 128-144 of the PAK pilin (Figure 3-2-A). Although both antibodies bound to C-terminal peptides containing amino acid residues 128-144, the affinities for these peptides were exceptionally different. MAb PK99H had an approximately 1000 fold higher titre for PAK(128-144)ox when compared directly to MAb PKL1 (Figure 3-2-B). The MAb PKL1 endpoint titre for the PAK(128-144)ox peptide

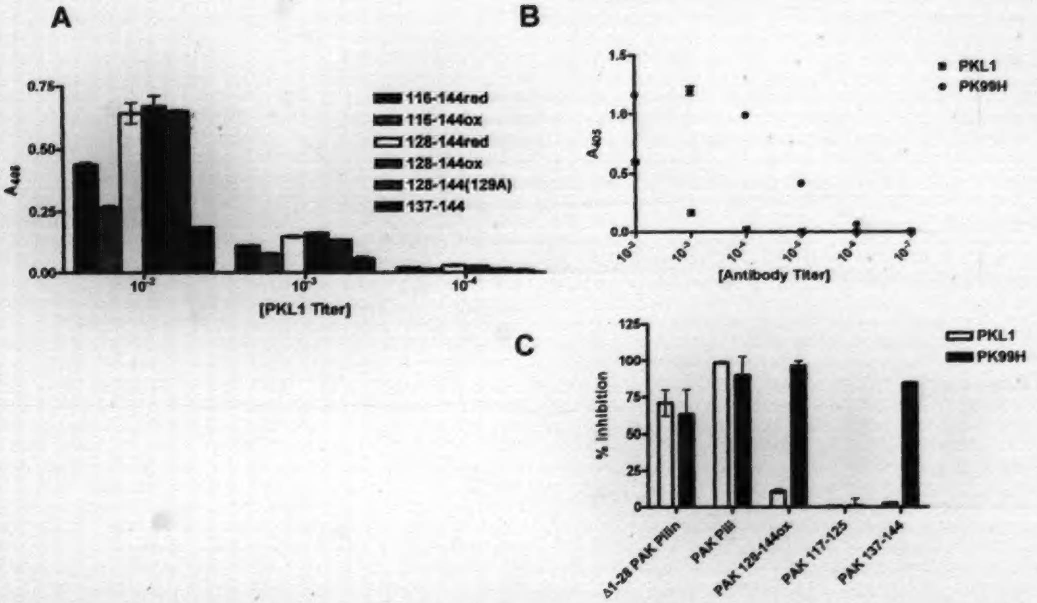


Figure 3-2. (A) Direct ELISA using PAK pilin peptides from the C-terminal receptor binding domain conjugated to BSA. PAK pilin peptides were coated into 96 well polystyrene plates at a concentration of 5 µg/mL. PAK pilin peptides were detected using PKL1. The development used rabbit anti-mouse 2° antibodies conjugated to horseradish peroxidase and ABTS. The absorbance readings were taken at 405nm. The results represent data from 3 independent experiments done in duplicate and error bars represent SEM. (B) Direct ELISA using PAK(128-144)ox conjugated to BSA. PAK(128-144)ox was coated into 96 well polystyrene plates at a concentration of 5 µg/mL. The PAK(128-144)ox BSA conjugated peptide was detected using PKL1 and PK99H. The development used rabbit anti-mouse 2° antibodies conjugated to horseradish peroxidase and ABTS. The absorbance readings were taken at 405nm. The results represent data from 2 independent experiments done in triplicate and error bars represent SEM. (C) Competitive ELISA assay using purified protein and PAK pilin peptides. Purified PAK pili were coated into 96 well polystyrene plates at a concentration of 5 µg/mL. PKL1 and PK99H were pre-incubated with 5 µg/mL PAK pilin, PAK pili or 25 µg/mL PAK 128-144ox, PAK 117-125, or PAK 137-144 for 30 minutes. Monoclonal antibody mixtures were added to the PAK pili coated plates. The development used rabbit anti-mouse 2° antibodies conjugated to horseradish peroxidase and ABTS. The absorbance readings were taken at 405nm. The results represent the data from 3 independent experiments performed in triplicate and error bars represent SEM.

was also approximately 1000 fold lower than the endpoint titres observed for PAK pili and PAK pilin monomer (compare Figure 3-1-B and 3-2-B). Neither antibody bound to any of the other peptides present in the PAK pilin (data not shown). As both monoclonal antibodies had affinities for the C-terminal peptides competitive ELISAs with PAK pili, PAK pilin, PAK(128-144)ox, PAK(117-125), and PAK(137-144) against immobilized PAK pili were performed to determine if these interactions were specific and physiologically relevant (Figure 3-2-C). The C-terminal peptides PAK(128-144)ox and PAK(137-144) were able to inhibit the binding of MAb PK99H to PAK pili as expected, confirming the specificity of this interaction (Figure 3-2-C). Also, in agreement with the data presented in Figure 3-1-B, both PAK pili and PAK pilin were able to inhibit MAb PK99H binding to PAK pili. None of the peptides tested had any effect on the binding of MAb PKL1 to PAK pili (Figure 3-1-B). Therefore, as MAb PKL1 has a low affinity for C-terminal peptides and a very high affinity for the monomeric pilin, the MAb PKL1 epitope must be a non-linear epitope involving a 3-dimensional structure near the C-terminal receptor binding domain of PAK pilin.

3. 3. 3. MAb PKL1 Inhibits MAb PK99H epitope recognition

The monoclonal antibodies MAb PK99H and MAb PKL1 do not have the same epitope specificity, even though MAb PKL1 has some affinity for the linear sequences in the C-terminal RBD (Figure 3-2-A) (Yu *et al.*, 1994). As the MAb PKL1 titre for the intact PAK pilus was three orders of magnitude higher than the titre for the PAK(128-144)ox peptide, the MAb PKL1 should bind to a non-linear epitope present in the PAK pilin subunit (Figures 3-1-B and 3-2-B). However, it is possible that both monoclonal antibodies bind to epitopes that are present at the tip of the PAK pilus. Sandwich ELISAs were used to determine if MAb PKL1 could inhibit MAb PK99H epitope recognition of PAK pili or vice versa. Both MAb PKL1 and MAb PK99H were able to inhibit epitope recognition by homologous monoclonal antibodies (Figure 3-3). However, only MAb PKL1 could inhibit epitope recognition by MAb PK99H (Figure 3-3). Therefore, the inhibition observed cannot be due to steric interference as in this case, MAb PK99H would have also prevented MAb PKL1 binding. This suggests that either MAb PKL1 binds to more sites on the pilus than MAb PK99H or binding of MAb PKL1 to the pilus alters the MAb PK99H epitope.

3. 3. 4. MAb PKL1 binds close to or at the pilus tip and inhibits binding to several substrates

To determine if MAb PKL1 is able to bind to the tip of the pilus, cellular inhibition assays were used. MAb PKL1 was able to inhibit binding of PAK pili to both BECs (Figure 3-4-A) and to asialo-GM$_1$ (Figure 3-4-B). The polyclonal anti-PAK(128-144)ox rabbit sera was used as a positive control and as expected was able to inhibit binding to both BECs (Figure 3-4-A) and asialo-GM$_1$ (Figure 3-4-B). Interestingly, MAb PK99H did not produce significant inhibition of PAK pilus-mediated binding to either BECs (Figure 3-4-A) or asialo-GM$_1$ (Figure 3-4-B) at the antibody concentrations used. Therefore, MAb PKL1 is a much better inhibitor of PAK pilus-mediated cellular binding than MAb PK99H, which requires

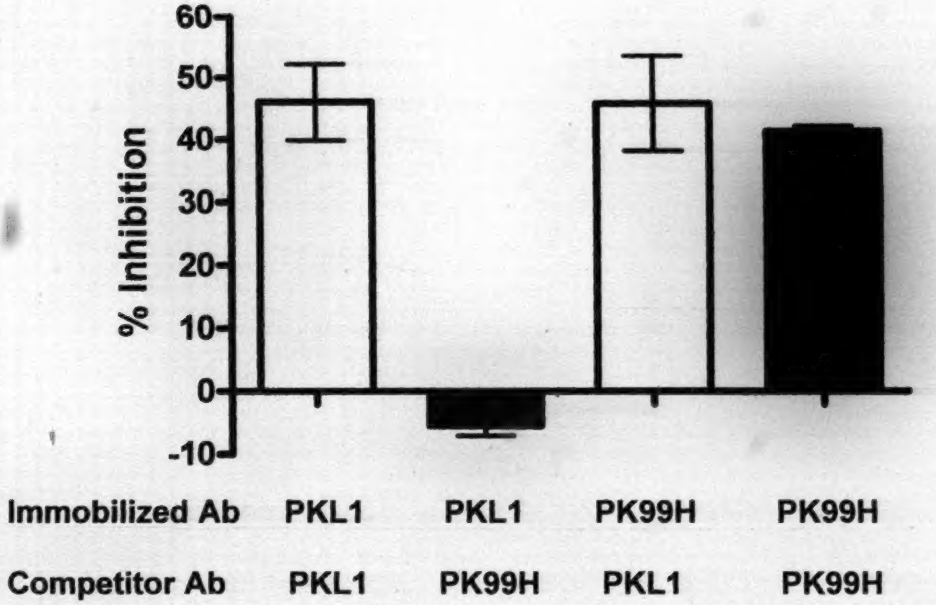


Figure 3-3. Indirect competitive ELISA assay using PKL1 and PK99H. Monoclonal antibodies PKL1 and PK99H were coated into 96 well polystyrene plates at concentrations of 5 µg/mL and 10 µg/mL respectively. Biotinylated purified PAK pili at 2.5 µg/mL were pre-incubated with 10^{-2} to 10^{-5} titers of PKL1 or PK99H for 30 minutes. Monoclonal antibody and PAK pili reaction mixtures were added to the PKL1 and PK99H plates. Biotinylated PAK pili were detected using streptavidin conjugated to horseradish peroxidase and ABTS. The absorbance readings were taken at 405nm. The results represent data collected from 2 independent experiments performed in triplicate and error bars represent SEM.

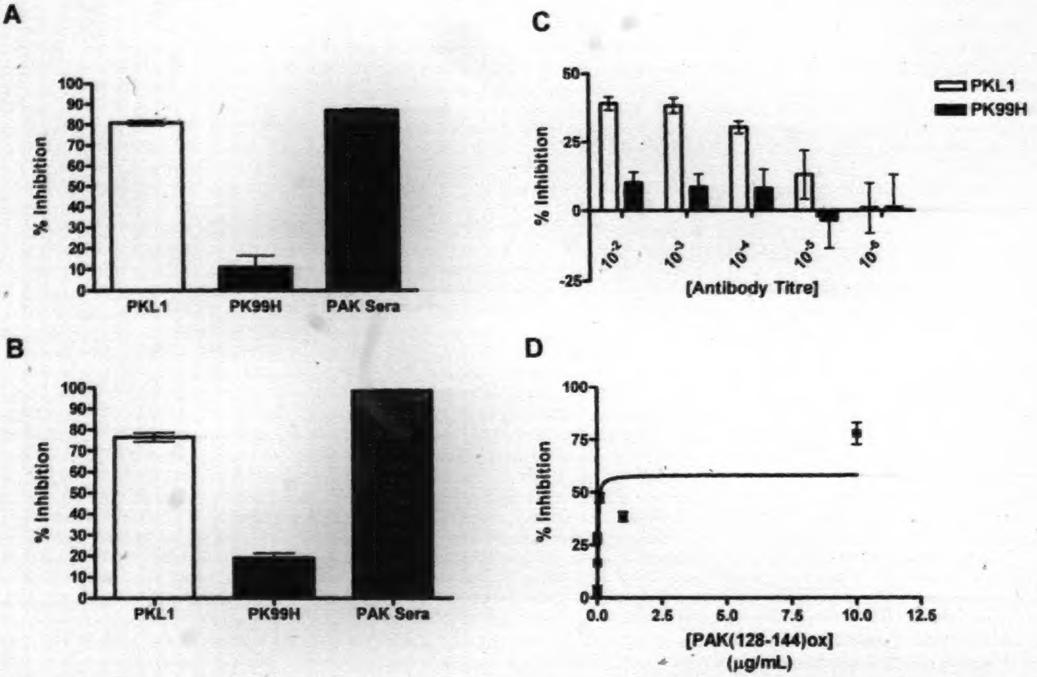


Figure 3-4. Inhibition of pilus-mediated cellular binding by monoclonal antibodies PKL1 and PK99H, or PAK(128-144)ox peptide. (A) PKL1, PK99H, or polyclonal anti-PAK(128-144)ox sera raised against the C-terminal RBD peptide (labeled PAK sera) were incubated with biotinylated purified PAK pili for 30 minutes at room temperature. Biotinylated PAK pili were then added to a BEC plate and incubated for 1 hour at 37°C. Plates were washed 3 times with buffer A and bound pili were detected using streptavidin conjugated horseradish peroxidase and ABTS. The absorbance readings were taken at 405nm. The results represent data collected from 3 independent experiments performed in triplicate and error bars represent SEM. (B) PK99H, PKL1, or polyclonal anti-PAK(128-144)ox sera were incubated with biotinylated purified PAK pili for 30 minutes at room temperature. Biotinylated PAK pili were then added to 96 well polystyrene plates containing immobilized asialo-GM_1 for 1 hour at 37°C. The plates were developed as described above. The absorbance readings were taken at 405nm. The results represent data collected from 3 independent experiments performed in triplicate and error bars represent SEM. (C) Inhibition of pilus-mediated binding to asialo-GM_1 using PK99H and PKL1 performed as described (Lee *et al.*, 1994). The results represent data from 2 independent experiments performed in triplicate and error bars represent SEM. (D) PAK(128-144)ox at various concentration was incubated with biotinylated PAK pili at a concentration of 0.1 µg/mL for 30 minutes and then added to a BEC plate for 1 hour at 37°C. The plates were developed as described above. The results represent data collected from 2 independent experiments performed in triplicate and error bars represent SEM.

very high antibody concentrations to cause significant inhibition (Doig *et al.*, 1990; Lee *et al.*, 1994). As the antibodies were titreed against PAK pili, MAb PKL1 and MAb PK99H are directly comparable in all assays used (Figure 3-1-B). Although these assay used whole IgG molecules, previous reports have determined that Fab fragments of both MAb PK99H and MAb PKL1 are still able to inhibit cellular binding (Doig *et al.*, 1990; Yu *et al.*, 1994).

It is interesting that even though MAb PK99H has a higher titre for amino acid residues 134-140 (and therefore the C-terminal RBD) this antibody was not able to inhibit cellular adherence by more than approximately 20% (Figures 3-4-A and 3-4-B). In contrast, MAb PKL1 at the same titre was able to inhibit cellular adherence by approximately 80% (Figures 3-4-A and 3-4-B). This assay was repeated using a different protocol with similar results (Figure 3-4-C) (Lee *et al.*, 1994). Potential concerns that MAb PK99H did not inhibit pilus-mediated cellular binding were addressed by performing a peptide inhibition assay using PAK(128-144)ox. In agreement with previous results this peptide was able to inhibit cellular adherence (Figure 3-4-D) (Irvin *et al.*, 1989). Therefore, MAb PKL1 likely binds at the pilus tip or close enough to the pilus tip to cause steric inhibition of cellular adherence.

To further examine binding activities of these monoclonal antibodies, the ability to inhibit DNA binding was tested. Our current hypothesis is that DNA binding involves amino acid residues displayed at the tip of the pilus and also along the solvent exposed length of the pilus (van Schaik *et al.*, 2005). In agreement with previous results MAb PK99H was able to inhibit pilus-mediated

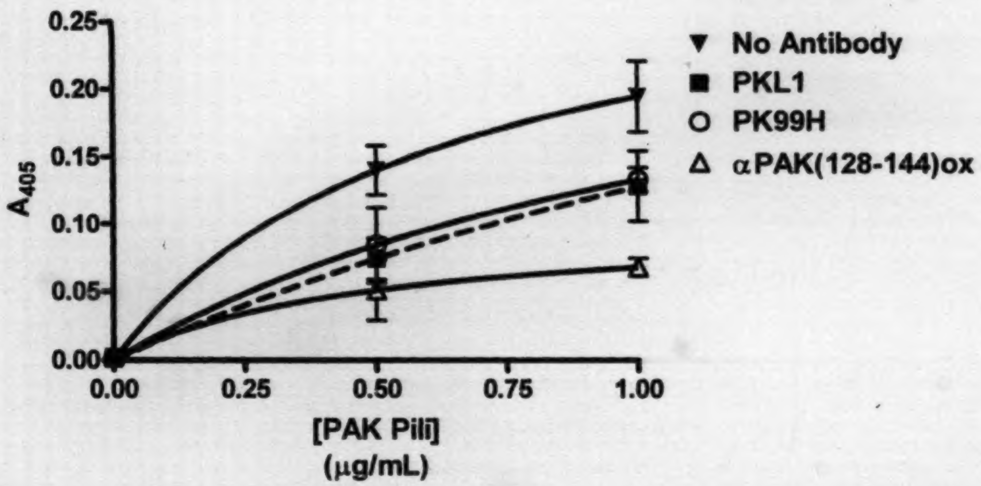


Figure 3-5. Inhibition of pilus-mediated DNA binding by monoclonal antibodies PKL1 and PK99H. PKL1, PK99H, or polyclonal anti-PAK(128-144)ox rabbit sera (labeled polyclonal PAK sera) raised against the C-terminal RBD peptide were pre-incubated with biotinylated PAK pili for 30 minutes at room temperature. Biotinylated PAK pili were then added to 96 well polystyrene plates containing immobilized pUCP19 plasmid DNA for 1.5 hour at room temperature. Plates were washed 3 times with buffer A and bound pili were detected using streptavidin conjugated horseradish peroxidase. The absorbance readings were taken at 405nm. The results represent two independent experiments performed in triplicate and error bars represent SEM.

DNA binding (Figure 3-5) (van Schaik *et al.*, 2005). In addition, MAb PKL1 monoclonal antibodies were also able to inhibit DNA binding at similar levels (Figure 3-5). This suggests that both MAb PKL1 and MAb PK99H can bind epitopes that are exposed at sites other than the tip, as pilus-mediated DNA binding is a multi-valent interaction (van Schaik *et al.*, 2005).

3. 3. 5. The C-terminal RBD is exposed at the tip and along the length of the PAK type IV pilus

Results from the current study and another recent study suggest that the C-terminal RBD is exposed along the length of the pilus, not just at the tip (Figure 3-5) (Smedley *et al.*, 2005). This is more consistent with evidence that suggests this domain is exposed along the length of the closely related *N. gonorrhoeae* pilus (Forest *et al.*, 1996). In addition, the results from the present study suggest that the MAb PK99H epitope is present at sites other than the tip of the pilus (Figure 3-1-B). Colloidal gold and electron microscopy were used to determine if this domain is in fact exposed along the length of the pilus. The majority of the antibodies from anti-PAK pili rabbit sera react with the 4-stranded anti-parallel β-sheet domain of the PAK pilin subunit exposed along the length of the pilus (Craig *et al.*, 2004; Hazes *et al.*, 2000; Sastry *et al.*, 1985b). In contrast, the majority of the antibodies from the anti-PAK(128-144)ox rabbit sera react with the C-terminal RBD of the PAK pilin subunit (Lee *et al.*, 1989b). To confirm these results a competitive ELISA was used to compete the PAK(128-144)ox peptide with immobilized PAK pili. This peptide was only able to inhibit the binding of the anti-PAK(128-144)ox sera to PAK pili (Figure 3-6-A). The PAK(128-144)ox peptide had no effect on the binding of the anti-PAK pili sera to PAK pili (Figure 3-6-A). Purified PAK pili were examined by transmission electron microscopy after treatment with anti-PAK pili rabbit sera or anti-PAK(128-144)ox rabbit sera followed by a gold-labeled secondary antibody. Figure 3-6-B confirmed that the β-sheet domain was exposed along the length of the pilus (Craig *et al.*, 2004; Hazes *et al.*, 2000). Interestingly, the anti-PAK(128-144)ox rabbit sera also bound along the length of the pilus, indicating that this domain is not only exposed at the tip of the pilus (Figure 3-6-C) (Lee *et al.*, 1994). This suggests that it is possible for MAb PK99H to bind sites exposed along the length of the pilus in addition to the pilus tip.

3. 3. 6. Antibodies specific for the C-terminal RBD can detect PAK pili bound to cellular receptors

The colloidal gold electron microscopy results demonstrate that the C-terminal RBD was exposed along the length of the PAK pilus (Figure 3-6-C). This indicates that MAb PK99H may bind to other sites in addition to the epitope exposed at the tip of the pilus. This was also indicated by the MAb PK99H titre against PAK pili and PAK pilin (Figure 3-1-B). The DNA inhibition results further supported the hypothesis that the MAb PK99H epitope is present at sites other than the tip of the pilus (Figure 3-5). To determine if MAb PK99H can bind to sites other than the pilus tip, and to determine if the MAb PKL1 epitope was present at other sites than the pilus tip cellular adherence assays were

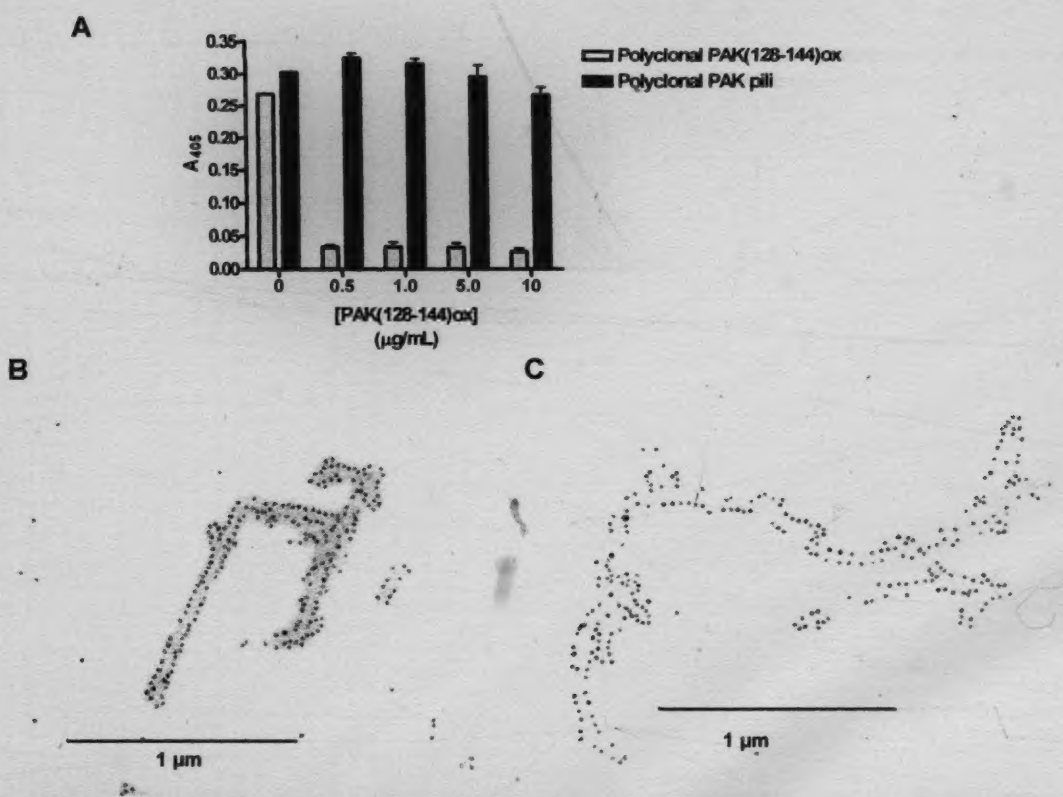


Figure 3-6. The C-terminal RBD is exposed along the length of the PAK pilus not just at the tip. (A) Competitive ELISA assay using PAK(128-144)ox peptide. Purified PAK pili were coated into 96 well polystyrene plates at a concentration of 5 µg/mL. Polyclonal anti-PAK(128-144)ox or polyclonal anti-PAK pili rabbit sera was pre-incubated with varying concentrations of PAK(128-144)ox for 30 minutes. The antibody-peptide mixtures were added to the PAK pili coated plates. The development used goat anti-rabbit 2° antibodies conjugated to horseradish peroxidase and ABTS. The absorbance readings were taken at 405nm. The results represent the data from 2 independent experiments performed in triplicate and error bars represent SEM. (B) Transmission electron micrograph of purified PAK pili probed with polyclonal anti-PAK pili rabbit sera and 20 nm gold-labeled 2° antibody. (C) Transmission electron micrograph of purified PAK pili probed with polyclonal anti-PAK(128-144)ox rabbit sera and 20 nm gold-labeled 2° antibody.

performed. As cellular adherence occurs at the tip of the pilus (Lee *et al.*, 1994), if the MAb PK99H or MAb PKL1 epitopes are exposed at other sites on the pilus, PAK pili bound to cellular receptors can be detected using MAb PK99H, MAb PKL1 or the polyclonal anti-PAK(128-144)ox rabbit sera. Alternatively, if the MAb PK99H or MAb PKL1 epitopes are only exposed at the tip of the pilus then these monoclonal antibodies would not be able to detect PAK pili bound to cellular receptors. This is possible since previous results have demonstrated that the base of the pilus is structurally different from the tip, and that the C-terminal RBD is probably not displayed at the base of the pilus (Lee *et al.*, 1994).

Purified PAK pili were allowed to bind to asialo-GM_1 (Figure 3-7-A) or BECs (Figure 3-7-B) followed by detection of the bound PAK pili with MAb PK99H, MAb PKL1, and polyclonal anti-PAK(128-144)ox sera. All of the antibodies tested were able to detect bound PAK pili on both asialo-GM_1 (Figure 3-7-A) and BECs (Figure 3-7-B). MAb PKL1 and the polyclonal anti-PAK(128-144)ox sera were able to detect similar amounts of bound PAK pili, while MAb PK99H detected smaller amounts of bound PAK pili (Figure 3-7-A and 3-7-B). In addition, the ability to detect viable *P. aeruginosa* PAK bacteria was tested after adherence to BECs. This should ensure that the C-terminal RBD was not just exposed at the base of the purified pili and account for any structural damage that may have occurred due to the purification process. In agreement with the data obtained with purified PAK pili, all antibodies were able to detect PAK cells bound to BECs (Figure 3-7-C). Again, MAb PKL1 and the polyclonal anti-PAK(128-144)ox sera were able to detect similar amounts of bound pili, whereas MAb PK99H detected

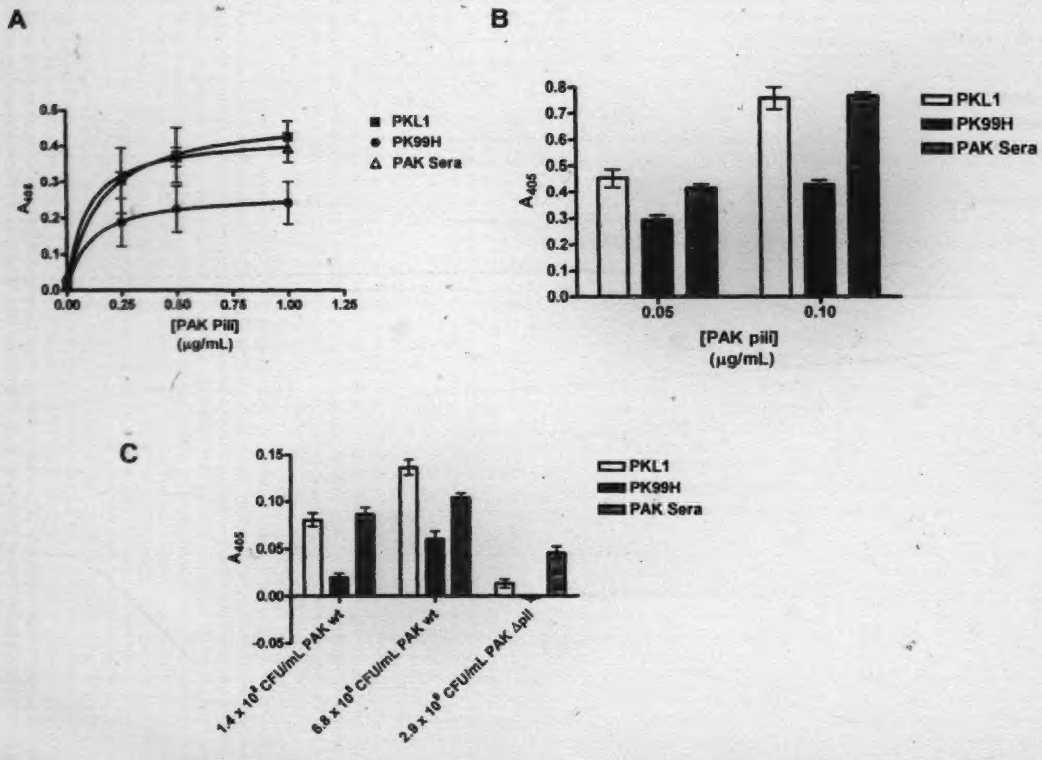


Figure 3-7. Detection of PAK pili bound to cellular receptors using monoclonal antibodies specific for the C-terminal RBD. (A) Purified PAK pili were incubated in 96 well polystyrene plates containing immobilized asialo-GM$_1$ for 1 hour at 37°C. The bound PAK pili were detected using PKL1, PK99H, or polyclonal anti-PAK(128-144)ox rabbit sera (labeled PAK sera). Antibodies were detected using either rabbit anti-mouse 2nd or goat anti-rabbit 2nd antibodies conjugated to horseradish peroxidase. The absorbance readings were taken at 405nm. The results represent data collected from 2 independent experiments performed in triplicate and error bars represent SEM. (B) Purified PAK pili were incubated in plates containing immobilized BECs for 1 hour at 37°C. The bound PAK pili were detected using PKL1, PK99H, or polyclonal anti-PAK(128-144)ox rabbit sera as described above. The absorbance readings were taken at 405nm. Results represent data collected from 2 independent experiments performed in triplicate and error bars represent SEM. (C) PAK wildtype or PAKΔpil a pilus deficient strain were added to plates containing immobilized BECs and developed as described above. The results represent the data from 1 experiment performed in triplicate. As there was such a difference in the number of CFU/mL between independent experiments the results could not be combined. However, similar results were observed in 3 independent experiments.

smaller amounts of bound pili (Figure 3-7-C). These results confirm that the C-terminal RBD (including residues 134-140) must be exposed at sites other than the pilus tip. In addition, the MAb PKL1 non-linear epitope is displayed at or close to the PAK pilus tip and at other sites along the solvent exposed surface of the pilus.

3. 4. Discussion

3. 4. 1. MAb PKL1 epitope specificity

Antibody epitopes can either be continuous linear peptides or discontinuous, where several discontinuous linear sequences from the primary structure interact to a form a 3-dimensional site (Berzofsky, 1985; Sela, 1969). This study further characterized the specificity of the monoclonal antibody MAb PKL1 originally constructed by Yu *et al.* (1994). As MAb PKL1 had the same affinity for both PAK pili and PAK pilin, the MAb PKL1 epitope is not a quaternary epitope present only in the intact pilus structure (Figure 3-1-B). Consequently, the MAb PKL1 epitope must be present in the PAK pilin monomer either as a linear epitope or as a discontinuous epitope. MAb PKL1 has some affinity for the linear PAK pilin peptides containing the C-terminal RBD, suggesting that the MAb PKL1 epitope could be a linear epitope (Figure 3-2-A). However, competitive ELISAs determined that this interaction was non-specific as the PAK C-terminal RBD peptides were unable to compete with PAK pili (Figure 3-2-C). In contrast, MAb PK99H bound to any peptide containing amino acid residues 134-140 and was inhibited by similar peptides in agreement with previous results (Figures 3-2-B and 3-2-C) (Wong *et al.*, 1992). Therefore, MAb PKL1 and MAb PK99H do not have the same specificity, and the MAb PKL1 epitope must be a 3-dimensional epitope.

An explanation for the ability of MAb PKL1 to bind the C-terminal RBD peptides is that a region of the linear sequence from this domain may represent the immuno-dominant peptide from the discontinuous epitope in which case, MAb PKL1 would interact with this peptide in direct ELISAs (Gao and Esnouf, 1996). However, this conclusion does not agree with the ability of MAb PKL1 to inhibit the binding of MAb PK99H to PAK pili and the inability of MAb PK99H to inhibit binding of MAb PKL1 to PAK pili (Figure 3-3). If a linear sequence from the C-terminal RBD was part of the MAb PKL1 epitope then MAb PK99H should have inhibited the binding of MAb PKL1 to PAK pili. I propose that the MAb PKL1 epitope consists of a region of the 4 stranded β-sheet and the loop connecting β-strands two and three (Figure 3-8). This secondary structure is disrupted by cleavage of the PAK pilin with trypsin and would not be present in the linear peptides (Sastry *et al.*, 1985b; Watts *et al.*, 1983b). This agrees with previous results demonstrating that the β-sheet structure is immuno-dominant in the intact pilus (Sastry *et al.*, 1985b; Watts *et al.*, 1983b). The binding of MAb PKL1 to the loop adjacent to the C-terminal RBD may alter the structure of the RBD, which would inhibit the ability of MAb PK99H to bind. NMR studies have determined that both loops are flexible in the K122-4 pilin subunit (Suh *et al.*, 2001). Therefore, the possibility exists that binding of MAb PKL1 to the adjacent

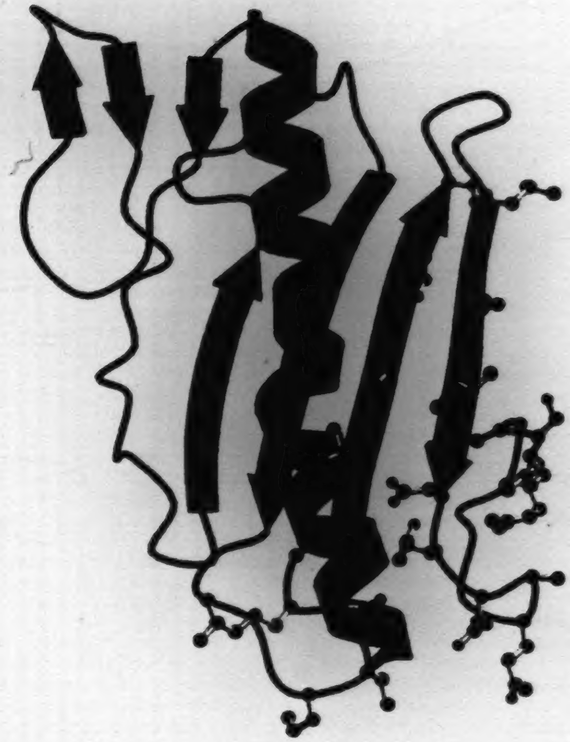


Figure 3-8. Structure of PAK monomeric pilin (Δ1-28) (Hazes *et al.*, 2000). This model of the truncated PAK pilin was designed using Molscript. The C-terminal disulfide bonded loop or the RBD is shown in red and a region of this loop, amino acid residues 134-140, is the specific linear epitope of PK99H (Wong *et al.*, 1992). The last two β-strands from the 4-stranded anti-parallel β-sheet, strands three and four are shown in green and a region of these strands is potentially part of the PKL1 3-dimensional epitope. The other structural aspect of the PAK pilin that creates a region of the 3-dimensional PKL1 epitope is probably the flexible loop connecting β-strands two and three shown in purple. The major antigenic determinants of the intact pilus structure are the 4-stranded β-sheet structure (Sastry *et al.*, 1985) and the C-terminal RBD (Lee *et al.*, 1989).

loop causes a structural change in the C-terminal RBD, which would prevent MAb PK99H epitope recognition. The potential ability of MAb PKL1 to change the conformation of the C-terminal RBD should be explored further to gain new insight into the binding mechanism of type IV pili.

Even though the MAb PKL1 epitope is not within the C-terminal RBD, this monoclonal antibody is still able to inhibit cellular binding (Figure 3-4). The prediction that the MAb PKL1 epitope lies within the adjacent loop and sections of the β-strands (Figure 3-8), may explain the observed inhibition of cellular binding through steric interference. Alternatively, binding of PKL1 may alter the C-terminal RBD so that the pilus is no longer capable of cellular adherence. Suprisingly, MAb PKL1 is a much better inhibitor of pilus-mediated cellular binding than MAb PK99H, which suggests that the MAb PKL1 epitope may be an excellent potential vaccine or therapeutic target (Figure 3-4). A method that could be used to determine the specific binding site of MAb PKL1 is to proteolytically cleave the PAK pilin after binding to MAb PKL1, followed by matrix-assisted laser desorption mass spectrometry (Parker *et al.*, 1996).

3. 4. 2. Implications for the structure and binding functions of the PAK type IV pilus

The colloidal gold transmission immuno-electron microscopy results demonstrate that the C-terminal RBD was displayed at sites other than the tip of the pilus (Figure 3-6-C). Therefore, analogous to the type IV pilus of *N. gonorrhoeae*, the C-terminal disulfide bonded loop of the *P. aeruginosa* pilin is displayed along the shaft of the pilus (Forest *et al.*, 1996). Indirect evidence to support the conclusion that the C-terminal RBD is exposed along the length of the pilus has also been presented in this study. The monoclonal antibody MAb PK99H was able to detect PAK pili or *P. aeruginsa* cells that were attached to BECs or asialo-GM$_1$ (Figure 3-7). Although it could be argued that detection of pili on whole *P. aeruginosa* cells may represent detection of unbound pili, the ELISA and detection results with purified PAK pili support the conclusion that the C-terminal RBD is displayed at sites other than the tip of the pilus.

Although this C-terminal RBD is solvent exposed along the shaft of the pilus, there is considerable evidence that pilus-mediated binding to epithelial cells is a tip specific event. Binding to asialo-GM$_1$ occurs at the tip of the pilus (Lee *et al.*, 1994), *P. aeruginosa* bind end on to epithelial cells, and free pili do not agglutinate BECs (R.T. Irvin unpublished results). In addition, synthetic C-terminal RBD peptides are able to inhibit pilus-mediated binding to BECs (Figure 3-4-D) (Irvin *et al.*, 1989), which would not be possible if the receptor interaction occurred along the length of the pilus due to avidity issues.

Interestingly, another report used MAb PK99H to detect the binding of the C-terminal RBD peptide to BECs (Irvin *et al.*, 1989), which indicates that the attributes recognized by MAb PK99H are not directly involved in the binding to BECs. Therefore, it was not unexpected that MAb PK99H was such a poor inhibitor of cellular binding (Figure 3-4), considering the MAb PK99H epitope may not be essential for binding. Even though MAb PK99H did not inhibit cellular binding, the synthetic peptide of the C-terminal RBD was still able to inhibit pilus-mediated cellular adherence (Figure 3-4-D). This suggests that the mechanism of type IV pilus-mediated binding by the C-terminal RBD remains elusive.

The ability of MAb PK99H and MAb PKL1 to inhibit another pilus-mediated interaction, DNA binding was also examined. Pilus-mediated DNA binding is a multivalent interaction as pilin subunits are unable to bind DNA, and the synthetic C-terminal RBD peptide was unable to inhibit DNA binding (van Schaik *et al.*, 2005). However, previous results also suggested that the C-terminal RBD is important for pilus-mediated DNA binding (van Schaik *et al.*, 2005). Both MAb PKL1 and MAb PK99H antibodies were able to inhibit pilus-mediated DNA binding by approximately 50%, indicating that other structural aspects of the intact pilus structure must be involved (Figure 3-5). As MAb PKL1 and MAb PK99H do not have the same specificity, the only explanation for the ability of these two antibodies to cause the same amount of inhibition is through steric interference. Therefore, I propose that the C-terminal RBD is not intimately involved in pilus-mediated DNA binding. Therefore, DNA binding is potentially mediated by the most immunodominant region of the pilus, the 4-stranded anti-parallel β-sheet that forms a groove lined with residues that are able to mediate interactions to the DNA backbone (van Schaik *et al.*, 2005).

The evidence provided in the current study that the C-terminal RBD is exposed along the length of the PAK pilus supports the 3-start left-handed helix model proposed by Craig *et al.* (2004) (Figure 3-9). In this model the C-terminal RBD is exposed along the length of the pilus and at the tip but not the base of the pilus (Figure 3-9). The discrepancies between the current results and the

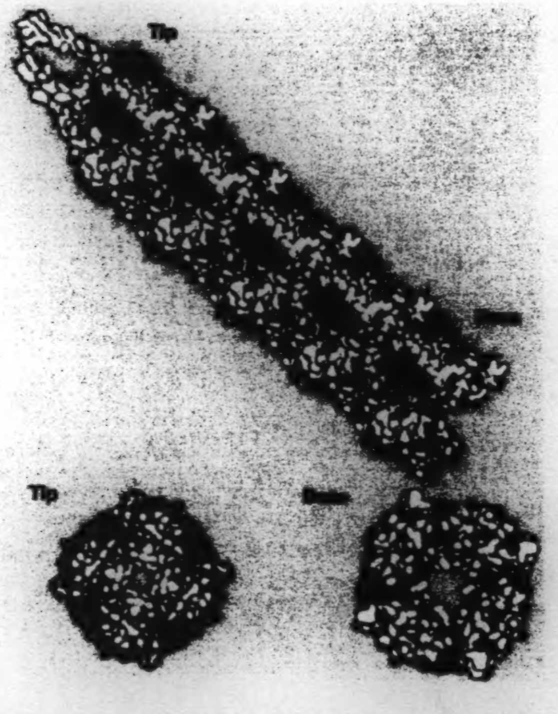


Figure 3-9. The 3-start left-handed helix model proposed by Craig *et al.* (2004). This model has been created with Web Viewer Pro using the full length PAK pilin monomer. The surface electrostatics are shown, **blue** represents positive, **red** negaitve, and **white** neutral charge. The N-terminal α-helices are shown in yellow and form the interior of the pilus. This model has been created with the hypothesis that the N-terminal α-helices are displayed at the tip of the pilus (Hazes *et al.*, 2000). In addition, the C-terminal RBD in green is displayed along the solvent exposed length of the pilus and multiple sites are present at the tip, but not the base of the pilus.

previous conclusion that the C-terminal RBD is only exposed at the tip of the pilus (Lee *et al.*, 1994) may reflect a clustering of RBDs at the pilus tip as opposed to actual surface exposure. This would also supports the 3-start left-handed helix model for the intact pilus (Figure 3-9) (Craig *et al.*, 2004).

Chapter 4

Quorum Sensing Autoinducers are Allosteric Regulators of *Pseudomonas aeruginosa* Type IV Pili, Which has Implications for Biofilm Formation

A version of this chapter is in preparation for submission to Molecular Microbiology. van Schaik, E.J., Giltner, C.L., Hassett, D.J., and Irvin, R.T.

C.L. Giltner performed many of the stainless steel assays and helped with revision of the manuscript. D.J. Hassett provided the synthetic autoinducers used in the above study.

4. 1. Introduction

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen, which can cause serious infections in cystic fibrosis, intensive care, burn, and immuno-compromised patients (Bodey *et al.*, 1983; Costerton, 2001; Pier, 1985; Speert, 2002). Initiation of an infection occurs after *P. aeruginosa* binds to host cells through several adhesins including type IV pili (Lyczak *et al.*, 2000b). Type IV pili are polar, filamentous protein structures made up of a single protein subunit PilA (Sastry *et al.*, 1985a). A complex type II secretion-like system that requires over 40 gene products is required for assembly of functional pili (Jacobs *et al.*, 2003; Peabody *et al.*, 2003; Wolfgang *et al.*, 2000). *P. aeruginosa* is able to bind to both biotic and abiotic surfaces through type IV pili (Craig *et al.*, 2004; Giltner *et al.*, 2006; Woods *et al.*, 1980). Interestingly, binding to a variety of substrates is mediated by the C-terminal receptor binding domain, or amino acid residues 128-144 of the PilA subunit (Giltner *et al.*, 2006; Irvin *et al.*, 1989; van Schaik *et al.*, 2005). Models of intact pili from several of the solved pilin structures all display the C-terminal receptor binding domain at the tip of the pilus where binding occurs (Audette *et al.*, 2004a; Craig *et al.*, 2004; Hazes *et al.*, 2000; Lee *et al.*, 1994; Parge *et al.*, 1995).

Attachment to surfaces by the tip of the type IV pilus allows for a flagella independent motility that requires extension and retraction of the pilus (Mattick, 2002). This motility, known as twitching motility, is dependent on several ATPases, including PilT, PilU, and PilB, which most likely generate the force required for assembly and retraction of the pilus (Kaiser, 2000; Merz *et al.*, 2000; Skerker and Berg, 2001).

The ability of type IV pili to mediate bacterial adherence and twitching motility permits the formation of biofilms (Klausen *et al.*, 2003a; Klausen *et al.*, 2003b; O'Toole and Kolter, 1998). Biofilms are complex surface associated structured bacterial communities (Costerton *et al.*, 1995a). *P. aeruginosa* produce many exopolymers, including exopolysaccharides, proteins, and DNA, that make up the extracellular polymeric substance matrix for the production of a structured biofilm (Friedman and Kolter, 2004b; Matsukawa and Greenberg, 2004b; Nemoto *et al.*, 2003; Whitchurch *et al.*, 2002). Biofilm formation occurs in response to quorum-sensing and is therefore dependent on *P. aeruginosa* population density (Davies *et al.*, 1998; Sauer *et al.*, 2002b).

Quorum sensing in *P. aeruginosa* is governed mainly by two intimately linked systems *las* and *rhl*. Each system is comprised of a transcriptional activator LasR or RhlR and an acyl-homoserine synthase LasI or RhlI, which synthesize the homoserine lactone signaling molecule needed for signal transduction (de Kievit and Iglewski, 2000; Pearson *et al.*, 1997; Venturi, 2006a). LasI is responsible for the synthesis of *N*-(3-oxododecanoyl)-L-homoserine lactone, or *P. aeruginosa* autoinducer-1 (PAI-1), while RhlI directs the production of *N*-butyryl-L-homoserine lactone, or *P. aeruginosa* autoinducer-2 (PAI-2) (Gambello and Iglewski, 1991b; Latifi *et al.*, 1995b; Winson *et al.*, 1995a). The autoinducers are released from *P. aeruginosa*; PAI-2 diffuses through the membrane, whereas PAI-1 is actively pumped out of the cell through multi-drug efflux pumps (Pearson *et al.*, 1999). When an appropriate cell density is reached these autoinducers enter *P. aeruginosa* at sufficient concentrations to regulate the expression of target genes.

This study describes a novel function of *P. aeruginosa* autoinducers (PAIs), the ability to modulate pilus-mediated binding mechanisms. Both PAI-1 and PAI-2 directly enhance the binding of *P. aeruginosa* type IV pili to stainless steel, DNA, and Buccal epithelial cells (BECs). The PAIs retain the ability to modulate pilus binding functions of cell surface associated pili on viable *P. aeruignosa*. However, the PAIs ability to modulate the adherence of *P. aeruginosa* cells is also dependent on the presence of functional PilT and PilU, which are ATPases involved in twitching motility. In addition, I investigated the effects of PAIs and DNA during the initial *P. aeruginosa* colonization of stainless steel as a model for biofilm formation. The presence of exogenous DNA can significantly increase the colonization of stainless steel in a type IV pilus dependent manner. The PAIs are also able to increase colonization in a pilus dependent manner, but in addition accelerate microcolony formation. In contrast, DNA can inhibit pilus-mediated binding to epithelial cells. Our results suggest that the DNA-pilus interaction is similar to an antibody-antigen interaction, where the ability to enhance or inhibit colonization depends on the number of *P. aeruginosa* cells and the concentration of DNA. I propose a novel function for the PAIs as allosteric regulators of *P. aeruginosa* type IV pili in addition to their role as ligands for quorum sensing transcriptional regulators during the quorum sensing response.

4. 2. Material and Methods

4. 2. 1. Bacterial strains, DNA, and PAIs

The *P. aeruginosa* strains used in this study were PAK a wild type strain, PAK 2Pfs a hyperpiliated PAK mutant (Bradley, 1974), PAK NP a pilus-deficient PAK mutant (Saiman *et al.*, 1990), PAKS34 a PAK twitching motility mutant (Whitchurch and Mattick, 1994) and PAKR364 another PAK twitching motility mutant (Whitchurch *et al.*, 1991). *P. aeruginosa* strains K122-4, PAO, and 1244, a strain that produces glycosylated type IV pili were also used in this study. *P. aeruginosa* were routinely grown at 37°C in Luria-Bertani (L-B) broth or LB supplemented with 150 µg/mL tetracycline (Sigma). PAK pili were purified from PAK 2Pfs as described (Paranchych *et al.*, 1979). The purity and integrity of the pili were assessed by 15% (w/v) SDS-PAGE and electron microscopy (Giltner *et al.*, 2006). *Escherichia coli* DH5α cells were used as the host cells for the purification of pUCP19 (Schweizer, 1991). *E. coli* DH5α harboring pUCP19 were cultured in LB supplemented with 100 µg/mL carbenicillin (Sigma), and pUCP19 was purified using the QIAfilter™ Giga Kit (Qiagen). In some instances, salmon sperm DNA was used as the eukaryotic DNA source (Roche). DNase I (Invitrogen) was used in direct binding assays or to degrade the salmon sperm DNA. The synthesis, purification and characterization of PAI-1 and PAI-2 have been reported elsewhere (Passador *et al.*, 1996). The C_{11}-SH hydrophobe (Sigma) was dissolved in 1-propanol and then diluted with methanol. Polyclonal rabbit anti-PAK pili sera used in this study has been reported previously (Sastry *et al.*, 1985b). Paired T-tests were used to compare differences between binding using the Prism 4 graphpad.

4. 2. 2. Biotinylation of *P. aeruginosa* cells and purified pili

Biotinylation of *P. aeruginosa* was preformed as described by Yu *et al.* (1996) with the following modifications. Harvested cells were suspended in 5 mL of phosphate buffered saline (0.01 M phosphate, 0.15 M NaCl) (PBS) pH 6.8 at an OD_{600} of 7.5 and incubated with 75 µl of 20 mg/mL biotinamidocaproate N-hydroxysuccinimidyl ester dissolved in dimethylsulfoxide at 22°C at 200 rpm in a water bath shaker for 55 minutes. The *P. aeruginosa* cells were washed four times by centrifugation at 10,000 x g for 10 minutes at 4°C before resuspension in PBS, pH 6.8 at an OD_{600} of 5.0. Viable counts were performed after biotinylation by serial dilution into PBS, pH 7.4 and plating 100 µL aliquots onto L-B agar. The procedure used for the biotinylation of the purified pili has been described previously (Yu *et al.*, 1996). The ability of the biotinylated pili to bind to asialo-GM_1 and GM_1 was determined as described by Lee *et al.* (1994) to confirm that the pili retained their functionality.

4. 2. 3. Stainless steel binding assays

Grade 304 stainless steel plates 1 mm thick and 7.6 by 11.5 cm were washed in 95% ethanol for 10 minutes, and rinsed with distilled water. Immediately before the binding studies, plates were washed with 20 mL of acetone for one minute with gentle agitation and rinsed with dH_2O. Plates were assembled into a

Schleicher and Schuell Manifold^{TM} System (Mandel Scientific). Biotinylated or unbiotinylated *P. aeruginosa* cells or purified, biotinylated PAK pili with or without a 45 minute pre-incubation with DNA, DNase I, or PAIs were added to the stainless steel manifold and incubated at 37°C for one hour. Alternatively, biotinylated *P. aeruginosa* cells were added to the stainless steel manifold and incubated for 30 minutes at 37°C. Varying concentrations of PAIs or the same volume of PBS, pH 7.4 were subsequently added to the *P. aeruginosa* cells and the manifolds were further incubated at 37°C for one hour. The manifold was subsequently washed five times with PBS, pH 7.4 containing 0.05 % (w/v) bovine serum albumin (BSA) (buffer A). Binding was quantified using streptavidin-HRP (Sigma) or polyclonal anti-PAK pili sera followed by secondary goat anti-rabbit HRP conjugate. All incubation times were one hour at 37°C followed by five washes with buffer A. Substrate buffer (0.01 M sodium citrate buffer pH 4.2 containing 1 mM 2,2'-Azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid] diammonium salt (ABTS) (Sigma) and 0.03% (v/v) hydrogen peroxide) was added to the manifolds and then incubated at room temperature (RT) for 10 minutes at 150 rpm. The absorbance was determined at 405 nm using a Multiskan Plus version 2.01 plate reader. Alternatively, after adherence of the *P. aeruginosa* cells, the steel plates were removed from the manifolds and stained with acridine orange 100 mg/mL for one minute and then washed twice for 10 minutes in dH_2O (Lauer *et al.*, 1981). The acridine orange stained steel plates were visualized using a Leitz K microscope equipped with epifluorescence and illumination using a 40x Neofluour lens. Micrographs were recorded using Kodak Colormax 35mm film in a MSP4 camera.

4. 2. 4. Cellular binding assays

Asialo-GM_1 and GM_1 immobilized receptor plates were prepared as described (Lee *et al.*, 1994). BEC plates were prepared by a described protocol with some minor modifications (Sexton and Reen, 1992). Tissue culture plates were incubated with 50 µg/mL poly-L-lysine in 0.01 M carbonate buffer pH 9.6 for two hours at 37°C and then washed twice with PBS, pH 6.8. The BECs were collected from healthy volunteers using wooden applicator sticks and were passed through 70 µm nylon mesh. BECs were adjusted to a concentration of 1 x 10^5 cells/mL determined by direct counts using a hemacytometer. The BECs were added to the 24 well tissue culture plates (Corning) (500 µL per well) and incubated for 45 minutes at 37°C. The BEC plates were centrifuged at 1000 x g for 10 minutes at RT and the unbound BECs were removed by aspiration. The BEC plates were then incubated with PBS, pH 6.8 over night at 4°C. The BEC plates were then blocked with 2 mg/mL gelatin in PBS, pH 6.8 for two hours at RT and then washed three times with PBS, pH 6.8. Biotinylated PAK pili were pre-incubated with varying concentrations PAI-1 and PAI-2, DNase I, or DNA for 45 minutes at RT before addition to the BEC or glycosphingolipid plates and further incubated at 37°C for one hour. Quantification of binding was performed as described in the stainless steel binding assay. DNA plates were prepared as described previously (van Schaik *et al.*, 2005). Biotinylated PAK pili were pre-incubated with PAI-1 and PAI-2 for 45 minutes at RT before addition to the DNA coated plates and then further incubated for 1.5 hours at RT. Quantification of binding was performed as described in the stainless steel assay. The direct binding to DNase I was performed by coating DNase I into 96 well polystyrene plates (Corning) at a concentration of 100 µg/mL in 0.01 M sodium carbonate buffer pH 9.5 over night at 4°C. The plates were washed three times with buffer A and then blocked with 5% (w/v) BSA overnight at 4°C. Biotinylated PAK cells were then added to the DNase I coated plates for one hour at 37°C. Quantification of binding was performed as described for the stainless steel assays.

4. 3. Results

4. 3. 1. *P. aeruginosa* autoinducers modulate cellular binding mediated by type IV pili

The quorum sensing PAIs from *P. aeruginosa* are involved in the regulation of many genes including a multitude of virulence genes (Brint and Ohman, 1995; Latifi *et al.*, 1995b). Previously, a novel function of the PAIs was proposed for *P. aeruginosa*: the control of twitching motility mediated by the type IV pili, as quorum sensing mutants were unable to twitch and had a reduction in number of surface assemblied pili (Glessner *et al.*, 1999). Although it was later determined that the PAIs do not control twitching motility as that the original mutants used for the above study had secondary mutations in *algR* and *vfr*, which affected twitching motility (Beatson *et al.*, 2002b). However, I decided to revisit the possibility that quorum sensing PAIs modulate type IV pilus functions independent of genetic regulation, as the PAIs did partially restore twitching motility in the quorum sensing mutants (Glessner *et al.*, 1999). Cellular binding by *P. aeruginosa* type IV pili is mediated by the C-terminal receptor binding domain present at the tip of the pilus (Lee *et al.*, 1994). Purified type IV pili were used to prevent ambiguous results due to genetic regulation or indirect modulation of cellular physiology.

The affects of PAI-1 and PAI-2 on the binding of type IV pili to BECs and the specific pilus receptor asialo-GM$_1$ were investigated. Both PAI-1 and PAI-2 increased the amount of binding to BECs when pre-incubated with purified type IV pili relative to binding in the absence of PAIs (Figure 4-1-A). The increase in type IV pili binding was concentration dependent and increased after a threshold PAI concentration of 6 μM was reached (Figure 4-1-A). These results are likely to be physiologically relevant, as physiological concentrations of PAIs produced by *P. aeruginosa* are >5 μM in stationary phase (Pearson *et al.*, 1995).

Interestingly, in contrast to the results obtained by pre-incubation of type IV pili with PAIs prior to BEC binding, only PAI-2 increased pilus-mediated binding to asialo-GM$_1$ (Figure 4-1-B). Similarly, the modulation of binding occurred more significantly after the threshold PAI-2 concentration of 6 μM was reached; however, the increase in binding to asialo-GM$_1$ was not strongly concentration dependent (Figure 4-1-B). Therefore, PAIs are able to directly increase the binding of *P. aeruginosa* pili to cellular receptors.

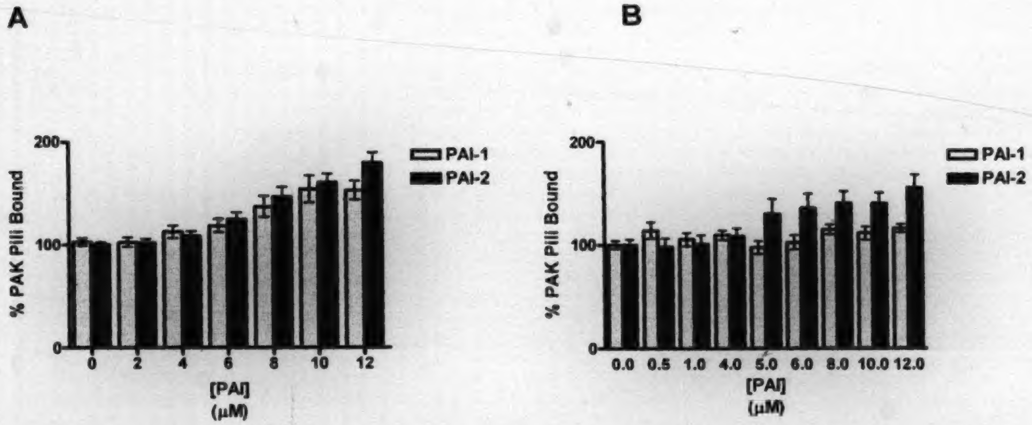


Figure 4-1. Exogenous synthetic PAIs increase pilus-mediated binding to BECs and asialo-GM$_1$ in a concentration dependent manner. (A) Biotinylated PAK pili at a concentration of 0.05 µg/mL were incubated with various concentrations of PAI-1 and PAI-2 for 45 minutes. The reactions were then added to BEC plates and incubated for one hour at 37°C. The plates were developed using SA-HRP and ABTS. The data represent four independent trials done in triplicate and the error bars represent SEM. (B) Biotinylated PAK pili at a concentration of 0.3 µg/mL were incubated with various concentrations of PAI-1 and PAI-2 for 45 minutes. The reactions were then added to asialo-GM$_1$ plates and incubated for one hour at 37°C. The plates were developed using SA-HRP and ABTS. The data represent two independent experiments performed in triplicate and the error bars represent SEM.

4. 3. 2. *P. aeruginosa* autoinducers modulate the binding of type IV pili to a variety of acellular substrates

The type IV pili of *P. aeruginosa* are able to mediate binding to substrates such as stainless steel, polyvinylchloride (Giltner *et al.*, 2006), and DNA (van Schaik *et al.*, 2005). Pilus-mediated binding to several of these substrates is mediated in part by the C-terminal receptor binding domain that is only available for binding at the tip of the pilus (Giltner *et al.*, 2006; Lee *et al.*, 1994). As PAI-1 and PAI-2 were both found to increase the binding of type IV pili to cellular receptors, the affects of the PAIs on binding to stainless steel and DNA were also investigated.

Both PAI-1 and PAI-2 enhanced the adherence of PAK cells (Figures 4-2-A, 4-2-C, and 4-2-E), but did not affect the adherence of PAK NP to stainless steel (a pilus-deficient isogenic mutant of PAK) (Figures 4-2-B, 4-2-D, and 4-2-F). This suggests that the increase is mediated by cell associated type IV pili. In addition, both PAIs significantly increased the binding of PAK cells (Figure 4-3-A) and specifically PAK pili (Figure 4-3-B) to stainless steel in a concentration dependent manner. This indicates that the increase in binding observed for purified pili is also apparent when using piliated wild type *P. aeruginosa* cells. An added effect due to pre-incubation of the PAK wild type cells with PAIs was the ability to accelerate microcolony formation (Figures 4-2-C and 4-2-E). The ability of a C_{11}-SH hydrophobe to modulate pilus-mediated binding to stainless steel was also tested as a control. This hydrophobe is a similar length to the acylated tail found on PAI-1. This hydrophobe caused a slight inhibition in the binding of both PAK

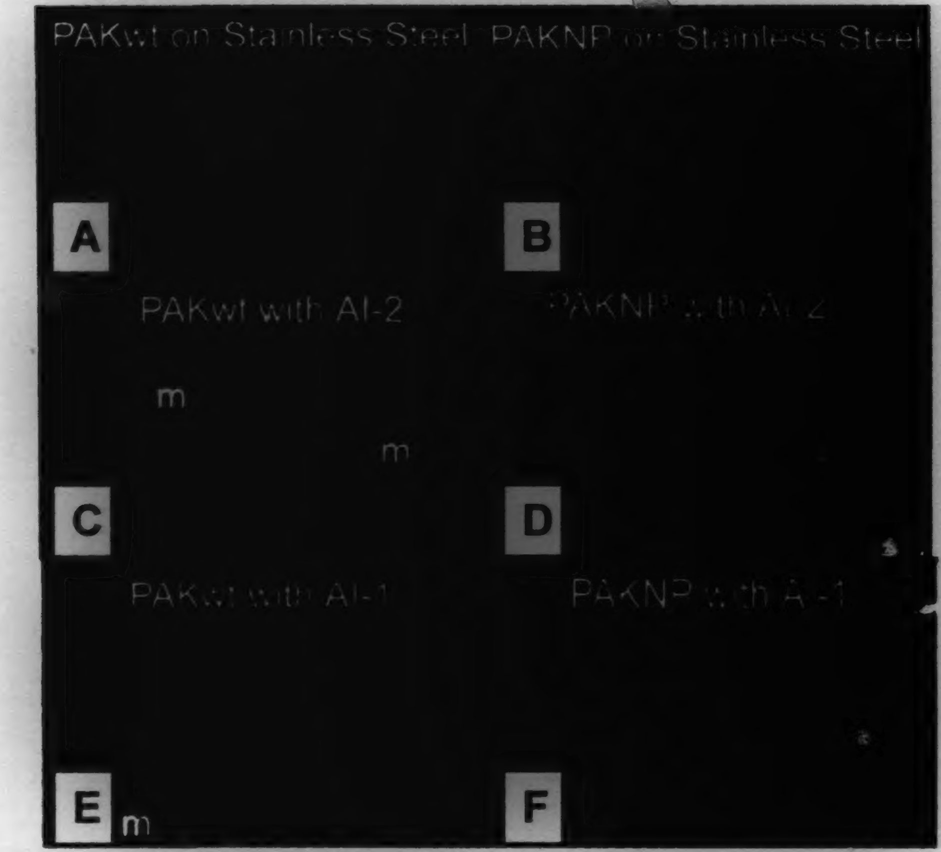

PAKwt on Stainless Steel
PAKNP on Stainless Steel
A
B
PAKwt with AI-2
PAKNP with AI-2
m
m
C
D
PAKwt with AI-1
PAKNP with AI-1
E
m
F

Figure 4-2. The effects of exogenous synthetic PAIs on the colonization of *P. aeruginosa* strains PAK and PAK NP to stainless steel. *P. aeruginosa* bacteria at a density of 1.1 x 10^{16} CFU/mL were pre-incubated with PBS pH 7.4 or PAIs at a concentration of 10 µM for 45 minutes. The reactions were then added to stainless steel manifolds and further incubated at 37°C for one hour. The plates were washed and stained with 100 mg/mL acridine orange. The acridine orange stained steel plates were visualized using a Leitz K microscope equipped with epifluorescence. Micrographs were recorded using Kodak Colormax 35mm film in a MSP4 camera. The *P. aeruginosa* cells appear orange and the green areas are non-specfic background staining. The m stands for microcolony.

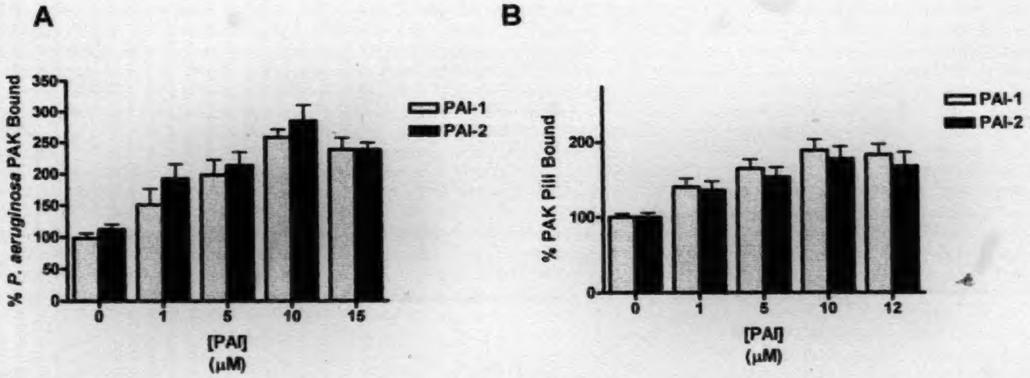


Figure 4-3. Exogenous synthetic PAIs increase the binding of *P. aeruginosa* PAK cells and purified pili to stainless steel in a concentration dependent manner. (A) Biotinylated *P. aeruginosa* PAK bacteria at a density of 1 x 10^8 CFU/mL were incubated with various concentrations of PAI-1 and PAI-2 for 45 minutes. The reactions were then added to stainless steel manifolds for one hour at 37°C. The plates were developed using SA-HRP and ABTS. The data represent two independent experiments performed in triplicate and the error bars represent SEM. (B) Biotinylated PAK pili at a concentration of 0.75 µg/mL were incubated with various concentration of PAI-1 and PAI-2 for 45 minutes. The reactions were then added to stainless steel manifolds for one hour at 37°C. The plates were developed using SA-HRP and ABTS. The data represent two independent experiments performed in triplicate and the error bars represent SEM.

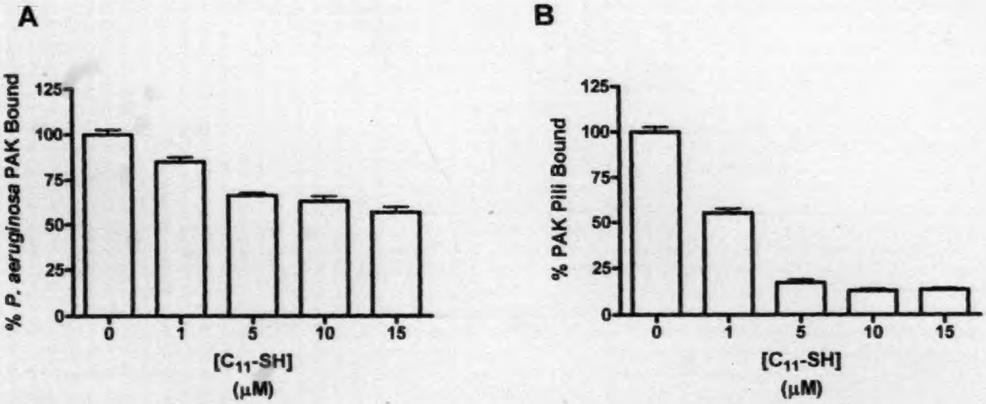


Figure 4-4. The effects of an exogenous hydrophobe on the binding of *P. aeruginosa* PAK cells or purified PAK pili to stainless steel. (A) *P. aeruginosa* PAK bacteria at a density of 2.3 x 10^8 CFU/mL were incubated with various concentrations of C_{11}-SH for 45 minutes. The reactions were then added to stainless steel manifolds and incubated at 37°C for one hour. The plates were developed using polyclonal anti-PAK pili rabbit sera and 2° HRP conjugated antibodies. The results represent two independent experiments performed in triplicate and the error bars represent SEM. (B) Biotinylated PAK pili at a concentration of 0.75 µg/mL were incubated with various concentrations of C_{11}-SH for 45 minutes. The reactions were then added to stainless steel manifolds for one hour at 37°C. The plates were developed using SA-HRP and ABTS. The data represent two independent experiments performed in triplicate and the error bars represent SEM.

cells (Figure 4-4-A) and PAK pili (Figure 4-4-B) to stainless steel, indicating that the observed PAI mediated increase is specific. Furthermore, the addition of similar amounts of methanol or ethanol to the binding reactions caused no difference in the binding of PAK pili to stainless steel (data not shown).

The effect of PAI-1 and PAI-2 on PAK pilus-mediated DNA binding was also investigated. PAI-1 and PAI-2 significantly enhanced the binding of biotinylated PAK pili to DNA in a concentration dependent manner that again occurred after a threshold concentration of 5 µM was reached (Figure 4-5). This is consistent with the results obtained for cellular binding (Figure 4-1). Therefore, in addition to increasing the adherence to cellular receptors, PAIs also increase pilus-mediated binding to acellular surfaces such as stainless steel and DNA.

4. 3. 3. The *P. aeruginosa* autoinducers do not modulate pilus-mediated binding of hyperpiliated twitching motility mutants

Twitching motility is a pilus dependent form of movement that involves cycles of extension and retraction of the type IV pili (Mattick, 2002). The extension and retraction of the pilus is mediated by several proteins that belong to a conserved family of ATPases (Patel and Latterich, 1998). Pilus extension is mediated by PilB, whereas pilus retraction is mediated by PilT and PilU (Lauer *et al.*, 1993; Nunn *et al.*, 1990; Whitchurch *et al.*, 1991; Whitchurch and Mattick, 1994). PAK R364 is a hyperpiliated, non-twitching strain of PAK created by insertion of Tn5-B21 into the *pilT* gene (Whitchurch *et al.*, 1991). PAK S34 is also a hyperpiliated, non-twitching strain of PAK with a Tn5-B21 insertion into the *pilU* gene

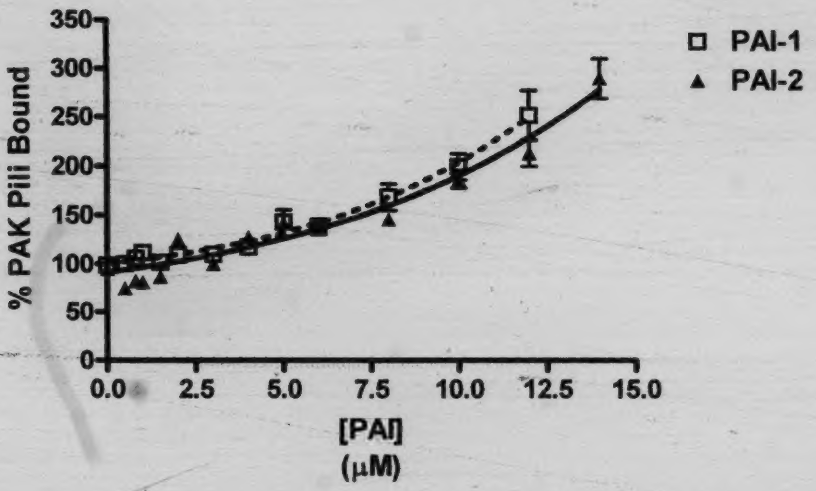


Figure 4-5. Exogenous PAIs increase pilus-mediated binding to immobilized DNA in a concentration dependent manner. Biotinylated PAK pili were incubated with various concentrations of PAI-1 and PAI-2 for 45 minutes. The reactions were then added to plates containing immobilized pUCP19 plasmid DNA and incubated at RT for 1.5 hours. The plates were developed using SA-HRP and ABTS. The data represent two independent experiments performed in triplicate and the error bars represent SEM.

(Whitchurch and Mattick, 1994). The ability of PAIs to alter the type IV pilus-mediated binding of these twitching motility mutants was examined.

Both R364 *pilT* and S34 *pilU* PAK mutants adhere to stainless steel in a concentration dependent manner similar to wild type PAK strain (data not shown) (Giltner *et al.*, 2006). Pre-incubation with PAI-1 or PAI-2 does not affect the adherence of R364 *pilT* and S34 *pilU* mutants to stainless steel, in contrast to wild type PAK where adherence is increased (compare Figures 4-6-A and 4-6-B with 4-3-A). Therefore, the responsiveness of the type IV pili from these twitching motility mutants to PAIs is altered.

The concentration of PAIs required for pilus modulation is only produced by *P. aeruginosa* at a "quorum" population, such as that formed by surface associated microcolonies during biofilm formation (Sauer *et al.*, 2002b). Consequently, I also investigated the affects of adding PAIs after *P. aeruginosa* had colonized the stainless steel surface. Wild type PAK cells detached from the stainless steel surface in the presence of physiological PAI concentrations by approximately 75% (Figure 4-7-A). However, R364 *pilT* mutants were unable to dissociate from the stainless steel surface after the addition of physiological concentrations of PAIs (Figure 4-7-B). Unexpectedly, there was an increase in the adherence of the S34 *pilU* mutant after colonization of the steel surface caused by the addition of physiological concentrations of PAIs (Figure 4-7-C). Therefore, the twitching motility ATPases seem to be partially required for the modulation of pilus functions by PAIs in intact *P. aeruginosa* cells.

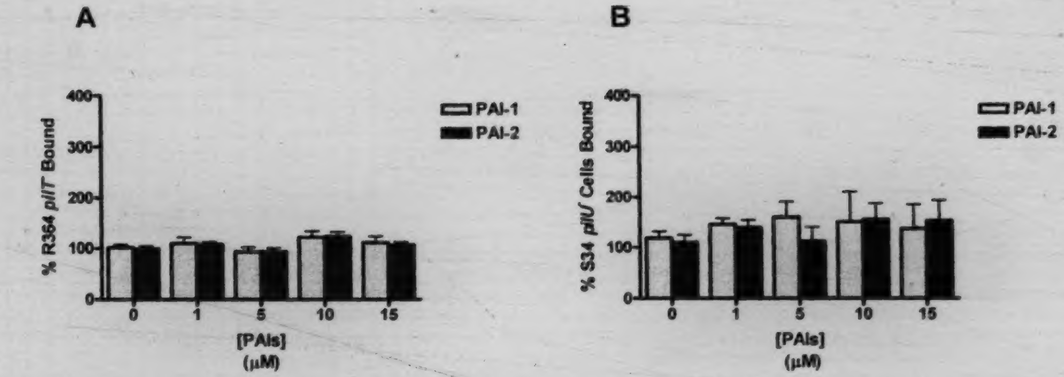


Figure 4-6. The effects of exogenous synthetic PAIs on the adherence of *P. aeruginosa* strains PAK R364 (Tn5-B21 into the *pilT* gene) or PAK S34 (Tn5-B21 insertion into the *pilU* gene) to stainless steel. (A) PAK R364 bacteria at a density of 2.4 x 10^6 CFU/mL were incubated with PAI-1 and PAI-2 for 45 minutes. The reactions were then added to stainless steel manifold and incubated at 37°C for one hour. The plates were developed using polyclonal anti-PAK pili rabbit sera and 2° HRP conjugated antibodies. The results represent two independent experiments performed in triplicate and the error bars represent SEM. (B) PAK S34 bacteria at a density of 2.1 x 10^6 CFU/mL were incubated with PAI-1 and PAI-2 for 45 minutes. The reactions were then added to stainless steel manifolds and incubated at 37°C for one hour. The plates were developed using polyclonal anti-PAK pili rabbit sera and 2° HRP conjugated antibodies. The results represent two independent experiments performed in triplicate and the error bars represent SEM.

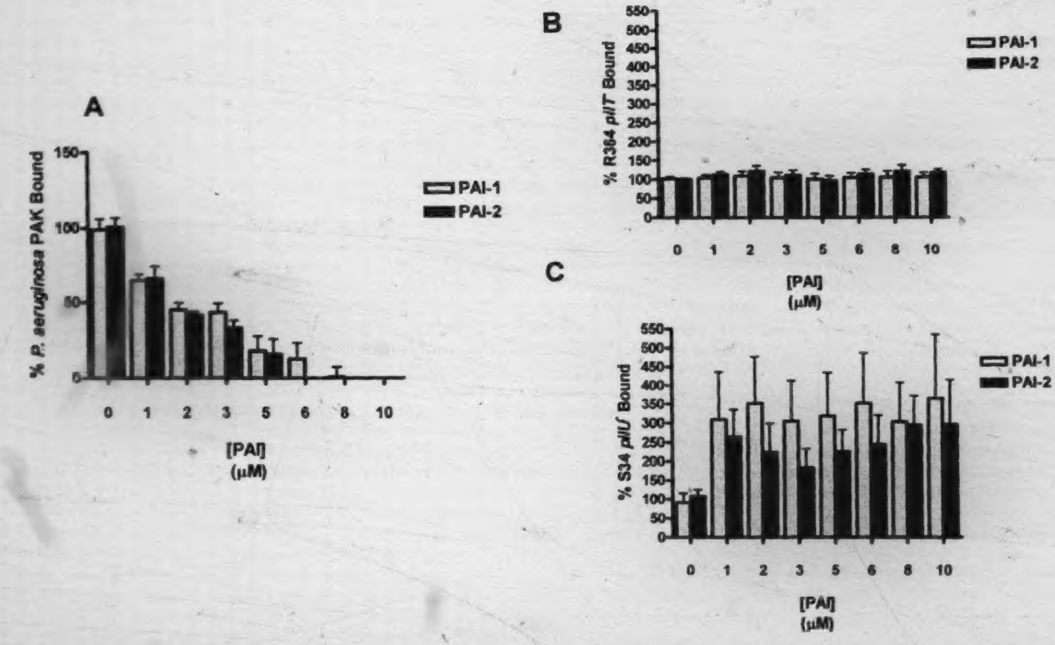
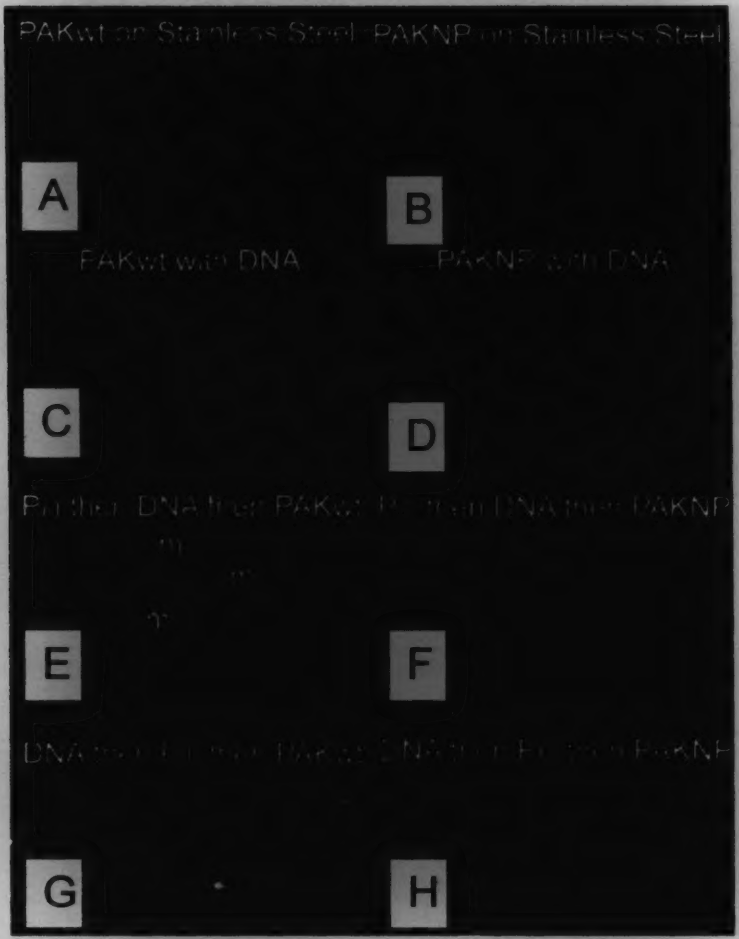


Figure 4-7. The effects of exogenous synthetic PAIs on *P. aeruginosa* PAK cells and isogenic mutants after colonization of stainless steel. (A) *P. aeruginosa* PAK bacteria at a density of 5.3 x 10^6 CFU/mL were added to the stainless steel manifolds and incubated for 30 minutes at 37°C. Varying concentrations of the PAIs or the same volume of PBS pH 7.4 were then added to the *P. aeruginosa* PAK cells and the manifolds were further incubated at 37°C for one hour. The plates were developed using polyclonal anti-PAK pili rabbit sera and 2° HRP conjugated antibodies. The results represent two independent experiments performed in triplicate and the error bars represent SEM. (B) PAK R364 bacteria at a density of 2.7 x 10^6 CFU/mL were added to stainless steel manifolds and developed as described above. (C) PAK S34 bacteria at a density of 2.3 x 10^6 CFU/mL were added to the stainless steel manifolds and developed as described above.

4. 3. 4. DNA affects the initial colonization of stainless steel and Buccal epithelial cells mediated by *P. aeruginosa* type IV pili

Several reports indicate that extracellular DNA is an important component of *P. aeruginosa* biofilms as DNases can disassociate young biofilms (Nemoto *et al.*, 2003; Whitchurch *et al.*, 2002). Although it is well established that DNA is involved in biofilm formation by *P. aeruginosa* it is not known whether DNA contributes to the initial colonization of surfaces or what role DNA plays during biofilm formation. As it was previously observed that sequence-independent DNA binding is mediated by the *P. aeruginosa* type IV pilus (van Schaik *et al.*, 2005), the effect of adding DNA during the colonization of stainless steel was examined. Exogenous DNA increased the colonization of PAK cells (Figures 4-8-A and 4-8-C), but did not enhance the ability of PAK NP (Figures 4-8-B and 4-8-D) to colonize stainless steel. This suggested that pilus-mediated DNA binding caused an increase in the colonization of *P. aeruginosa* to stainless steel (van Schaik *et al.*, 2005). The addition of exogenous DNA also increased the adherence of *P. aeruginosa* K122-4 and 1244 (Figure 4-9-A) to stainless steel in a concentration-dependent manner, indicating that the increased adherence is not specific for PAK cells or dependent on the formation of non-glycosylated pili. However, this increase was dependent on the number of *P. aeruginosa* cells and the concentration of DNA. Although the visual aggregation and increase in colonization of stainless steel was always observed, certain combinations of *P. aeruginosa* cells and DNA produced no quantitative change or an inhibition in colonization (data not shown). In addition, there was no apparent increase in



PAKwt on Stainless Steel
PAKNP on Stainless Steel
A
B
PAKwt with DNA
PAKNP with DNA
C
D
Purified DNA from PAKwt
Purified DNA from PAKNP
m
E
F
G
H

Figure 4-8. The effects of exogenous DNA on the colonization of *P. aeruginosa* strains PAK and PAK NP to stainless steel. *P. aeruginosa* bacteria at a density of 9.5 x 10^{16} CFU/mL were pre-incubated with PBS pH 7.4 or DNA at a concentration of 15 mg/mL for 45 minutes. The reactions were then added to stainless steel manifolds and further incubated at 37°C for one hour. The plates were washed and stained with 100 mg/mL acridine orange. The acridine orange stained steel plates were visualized using a Leitz K microscope equipped with epifluorescence. Micrographs were recorded using Kodak Colormax 35mm film in a MSP4 camera. The *P. aeruginosa* cells appear orange and the green areas are non-specfic background staining. The m stands for microcolony.

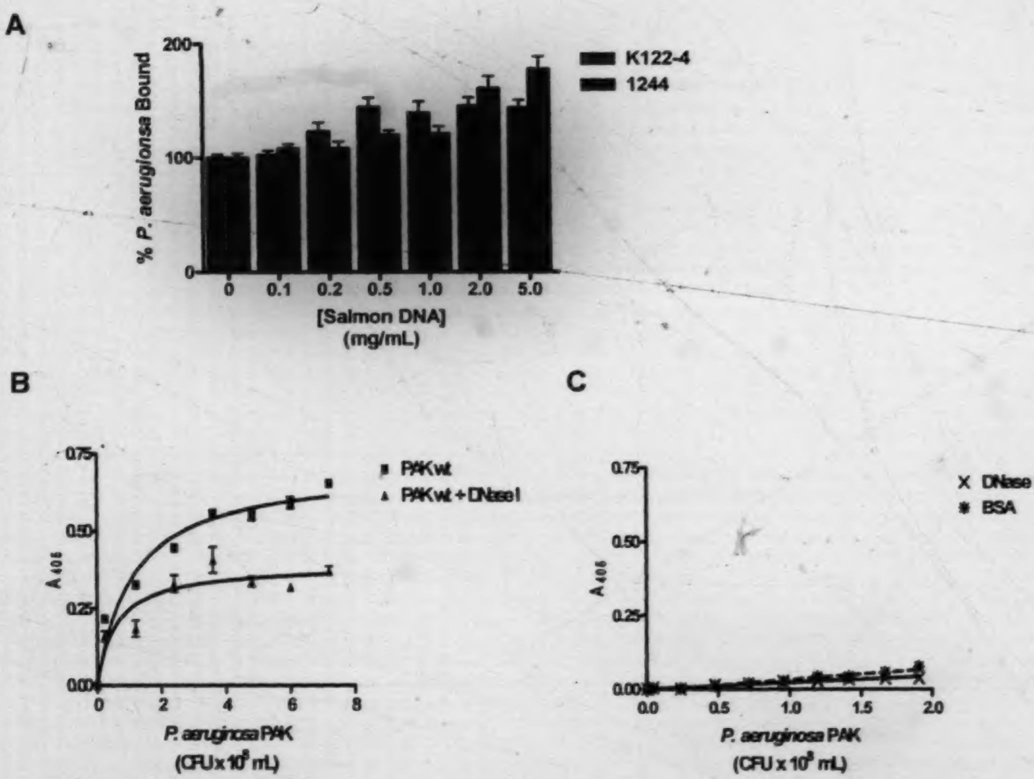


Figure 4-9. Exogenous DNA increases the *P. aeruginosa* colonization of stainless steel. (A) Biotinylated *P. aeruginosa* K122-4 or 1244 bacteria at cellular densities of 5.8 x 10^6 CFU/mL and 6.7 x 10^6 CFU/mL respectively were incubated with various concentrations of DNA for 45 minutes. The reactions were then added to stainless steel manifolds for one hour at 37°C. The plates were developed using SA-HRP and ABTS. The data represent two independent experiments performed in triplicate and the error bars represent SEM. (B) Biotinylated *P. aeruginosa* PAK bacteria were added to the stainless steel manifolds in the presence of absence of 76 units of DNase I. The plates were developed as described above. (C) DNase I or BSA were immobilized into microtiter plates at a concentration of 100 µg/mL. Biotinylated *P. aeruginosa* PAK cells were added to the plates and incubated for one hour at 37°C. The plates were developed using SA-HRP and ABTS. The data represent two independent experiments performed in triplicate and the error bars represent SEM.

binding using purified PAK pili and exogenous DNA (data not shown). However, as the affinity of the type IV pilus for stainless steel is very high (Giltner *et al.*, 2006), this was not unexpected as very high concentrations of DNA may be required to cause an increase.

To confirm that the pilus-DNA interactions were responsible for the increase in colonization of stainless steel, an additional set of experiments were carried out. Purified pili were allowed to bind to the stainless steel, followed by the addition of exogenous DNA and finally PAK cells. Increased adherence of PAK cells (Figure 4-8-E), but not PAK NP cells to the stainless steel was observed (Figure 4-8-F). The reciprocal study, where DNA was first incubated with the steel followed by the addition of PAK pili and then the addition of PAK cells, virtually abolished the binding to stainless steel (Figure 4-8-G).

DNase I has been shown to prevent biofilm formation; this effect may in part be due to a decrease in the initial colonization of the surface (Nemoto *et al.*, 2003; Whitchurch *et al.*, 2002). Therefore, the effect of DNase I treatment on the adherence of *P. aeruginosa* PAK to stainless steel was investigated. The addition of DNase I caused a reduction in the binding of *P. aeruginosa* PAK to stainless steel (Figure 4-9-B), suggesting that exogenous DNA or *P. aeruginosa* released DNA (Allesen-Holm *et al.*, 2006; Kadurugamuwa and Beveridge, 1995) can increase the colonization to stainless steel. The absence of an interaction between *P. aeruginosa* PAK cells and DNase I provides evidence against this effect being caused by steric interference (Figure 4-9-C).

The effect of exogenous DNA on the colonization of BECs by P. aeruginosa was examined. Exogenous DNA at certain concentrations caused an inhibition in the ability of *P. aeruginosa* cells to adhere to BECs (Figure 4-10-A). Again, as with the colonization of stainless steel, the reduced adherence of *P. aeruginosa* cells to BECs was dependent on both the bacterial cell density and the concentration of DNA. The ability of DNA to increase or decrease pilus mediated binding to asialo-GM$_1$ was also investigated. Pre-incubation with exogenous DNA inhibited the specific interaction of PAK type IV pili with asialo-GM$_1$ by up to 75%, which is higher than the inhibition observed for *P. aeruginosa* cells (Figure 4-10-B). The ability of DNase I to inhibit pilus-mediated binding to BECs was also tested. DNase I was able to inhibit pilus mediated binding to BECs by ~25%, which was less than the inhibition observed for stainless steel (compare Figures 4-9-B and 4-10-C). To confirm that the DNA is mediating the observed inhibition, the DNA was treated with DNase I prior to the experiment. The DNase I treated DNA inhibited pilus-mediated binding by ~10% (Figure 4-10-C). However, the extent of DNA degradation was not quantified and therefore I can conclude that DNA specifically caused an inhibition of pilus-mediated binding to both BECs and asialo-GM$_1$. This suggests that the ability of DNA to aggregate *P. aeruginosa* cells (Allesen-Holm *et al.*, 2006) inhibits the colonization of BECs and increases the colonization of stainless steel. However, the ability of DNase I to inhibit binding to both BECs and stainless steel suggests that there are concentrations of DNA that will increase the colonization of *P. aeruginosa* for different substrates.

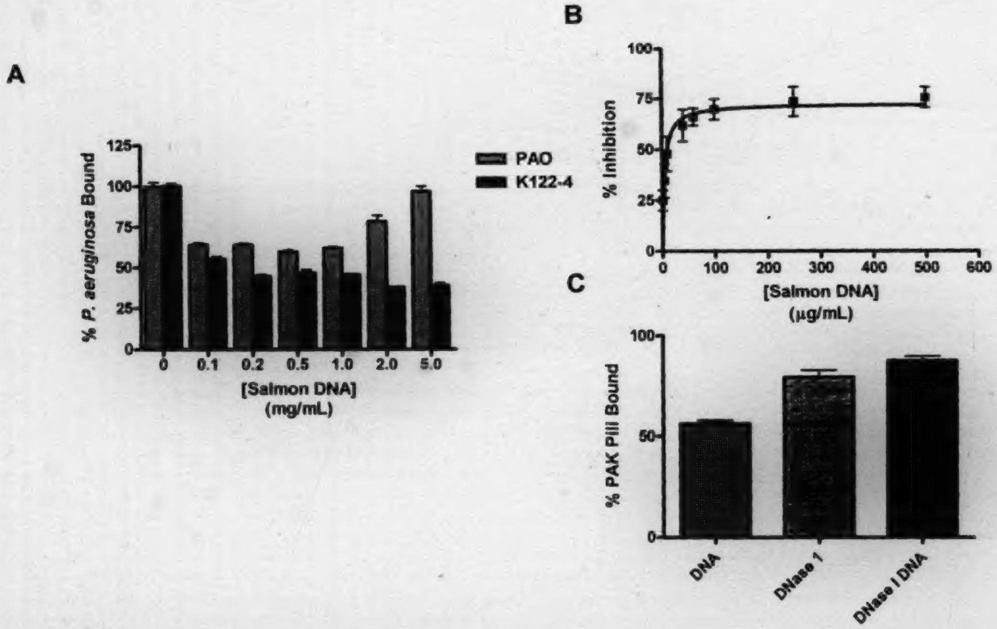


Figure 4-10. Exogenous DNA decreases the colonization of *P. aeruginosa* to BECs. (A) Biotinylated *P. aeruginosa* PAO or K122-4 bacteria at cellular densities of 9.7 x 10^6 CFU/mL and 5.9 x 10^6 CFU/mL respectively were incubated with various concentrations of DNA for 45 minutes. The reactions were then added to BEC plates and incubated for an hour at 37°C. The plates were developed using SA-HRP and ABTS. The data represent two independent experiments performed in triplicate and the error bars represent SEM. (B) Biotinylated PAK pili at a concentration of 0.3 µg/mL with various concentrations of DNA for 45 minutes. The reactions were then added to asialo-GM_1 plates and incubated for an hour at 37°C. The plates were developed using SA-HRP and ABTS. The data represent two independent experiments performed in triplicate and the error bars represent SEM. (C) Biotinylated PAK pili at a concentration of 0.05 µg/mL with 0.5 mg/mL DNA or DNase I treated DNA for 45 minutes. Alternatively, the biotinylated PAK pili were incubated with 56 units of DNase I for 45 minutes. The reactions were then added to BEC plates and incubated for an hour at 37°C. The plates were developed using SA-HRP and ABTS. The data represent two independent experiments performed in triplicate and the error bars represent SEM.

4. 4. Discussion

Biofilm formation by *P. aeruginosa* is of medical importance as biofilms form on a number of surfaces including the lungs of cystic fibrosis patients and indwelled catheters (Nickel *et al.*, 1989; Singh *et al.*, 2000). Initiation of biofilm formation by *P. aeruginosa* requires type IV pili and quorum-sensing (Davies *et al.*, 1998; O'Toole and Kolter, 1998). This report describes a novel function of *P. aeruginosa* quorum-sensing PAIs, the ability to modulate pilus-mediated binding to abiotic and biotic substrates. Both PAI-1 and PAI-2 produced by the Las and Rhl quorum-sensing systems respectively increase the binding of purified type IV pili to BECs (Figure 4-1-A). Although other cell adhesins have been identified in *P. aeruginosa* including alginate (Doig *et al.*, 1987; Mai *et al.*, 1993), LPS (Gupta *et al.*, 1994; Zaidi *et al.*, 1996), and flagella (Feldman *et al.*, 1998) mutations in the type IV pili cause the most drastic effect on adherence and account for approximately 90% of all host cell binding (Farinha *et al.*, 1994). It is therefore of significant interest that quorum-sensing PAIs increase the adherence of *P. aeruginosa* to host cells and may play a role during acute infections. However, as high cell densities are required to produce physiologically relevant PAI concentrations, it appears unlikely that PAIs would modulate the adherence of *P. aeruginosa* during the initial colonization preceeding infection (Davies *et al.*, 1998; Pearson *et al.*, 1995).

Exogenous synthetic PAI-1 and PAI-2 at physiological concentrations increased the adherence of wild type *P. aeruginosa* PAK cells expressing pili (Figures 4-2-A, 4-2-C, and 4-2-E), but not an isogenic PAK pilus-deficient mutant

(PAK NP) to stainless steel (Figures 4-2-B, 4-2-D, and 4-2-F). This increase in binding was concentration dependent for both PAK cells (Figure 4-3-A) and purified PAK pili (Figure 4-3-B). This suggests that the ability of PAIs to increase the binding of pili to BECs (Figure 4-1-A), stainless steel (Figure 4-3-B), and DNA (Figure 4-5) is not an artifact of pilus purification.

There are several possible explanations for the increase in pilus-mediated binding, the PAIs may (i) bind to the surface and facilitate colonization, (ii) cause aggregation of the pili, or (iii) bind to the pilus and increase the binding capacity. It is unlikely that both PAI-1 and PAI-2 would be able to bind to all of the substrates used (epithelial cells, stainless steel, and DNA) as the properties of these surfaces differ dramatically. In addition, the ability of the PAIs to increase pilus-mediated binding only above a threshold concentration of 6 μM is reached suggests an interaction of moderate affinity, whereas the pilus-steel interaction is of very high affinity and concentration dependent (Figure 4-1, 4-5). The ability of PAIs to alter the adherence of purified pili to the specific glycosphingolipid receptor asialo-GM_1 was also examined (Figure 4-1B). In agreement with the data obtained with BECs, PAI-2 was able to increase the binding of pili to asialo-GM_1 (Figure 4-1). In contrast, PAI-1 had no effect on the binding of pili to asialo-GM_1 despite increasing the binding of pili to BECs (Figure 4-1). These observations suggest: (i) There must be other pilus receptors present on the surface of BECs as determined previously (Doig *et al.*, 1989; Wu *et al.*, 1995), and PAI-1 is able to alter the affinity of the pilus for a subset of these receptors. and (ii) The increase in binding is not due to aggregation of the pili or PAI-1 would have also increased the binding of purified pili for asialo-GM$_1$ (Figure 4-1-B).

Therefore, the most likely basis for the increase in pilus-mediated binding to abiotic and biotic substrates is through direct binding of PAI-1 and PAI-2 to the pilus. Binding of either PAI-1 or PAI-2 likely induces a conformational change in the pilus that alters the C-terminal receptor binding domain structure. NMR studies using synthetic peptides of the C-terminal receptor binding domain or residues 128-144 have demonstrated that these peptides are found in multiple conformations (Campbell *et al.*, 1997; McInnes *et al.*, 1994; Wong *et al.*, 1995). This C-terminal receptor binding domain in the K122-4 pilin monomer has also been found to be very flexible in molecular dynamic studies (Suh *et al.*, 2001). Furthermore, comparisons of the solved truncated K122-4 structure by NMR (Keizer *et al.*, 2001) and X-ray crystallography (Audette *et al.*, 2004a) indicate that there may be multiple pilin conformations. Interestingly, exposure to a hydrophobe causes a conformational change in the truncated K122-4 pilin monomer that leads to spontaneous self-assembly into a protein nanotube (Audette *et al.*, 2004c). Therefore, the pilus structural subunit is capable of considerable flexibility.

The observation that PAIs modulate type IV pilus binding functions was not anticipated since the only well established function of these molecules is as ligands for the transcriptional regulators LasR and RhlR (Brint and Ohman, 1995; de Kievit and Iglewski, 2000; Ochsner and Reiser, 1995; Passador *et al.*, 1993; Pearson *et al.*, 1994). Ligands often act as "molecular switches" to cause an allosteric change in transcriptional factors to regulate gene expression (Buskirk and Liu, 2005). Therefore, I propose that in addition to their role in regulation of the transcriptional factors LasR and RhlR, the PAIs are also allosteric regulators of the binding functions mediated by the type IV pilus.

Nevertheless, the results using the twitching motility mutants suggested an additional level of control. Pre-incubation of PAI-1 and PAI-2 with R364 *pilT* or S34 *pilU* mutants did not cause an increase in adherence for stainless steel in contrast to the results obtained with PAK wild type cells (compare Figures 4-3-A with 4-6). This indicates a possible link between the potential conformational change induced in the pilus by the PAIs and the ATPases involved in twitching motility (Burrows, 2005). However, the ability of the PAIs to modulate or induce a conformational change in the purified pili is not compromised, as both PAI-1 and PAI-2 increase binding to several substrates (Figures 4-1-A, 4-3-B, and 4-5). This is interesting as the PAK pili were purified from the twitching motility mutant, PAK 2Pfs (Bradley, 1974; Paranchych *et al.*, 1979) indicating that the ability of the pili from the twitching motility mutants to respond to PAIs is not compromised. Purified pili have no base structure as they are sheared off of the *P. aeruginosa* cells (Paranchych *et al.*, 1979). Therefore, the pili displayed on the surface of *P. aeruginosa* have additional requirements to stabilize the conformational change induced by the PAIs. The base of the pilus is anchored to the inner membrane and potentially in transient contact with both PilT and PilU through a complex spanning the inner membrane (Burrows, 2005; Darzins and Russell, 1997). Therefore, it is possible that the ATPases act as secondary messengers to recognize and stabilize the conformational change induced by the PAIs. There is a conserved sequence (AIRNLIRE) in PilT may respond to pilus-mediated surface binding to signal retraction as mutations do not affect ATPase activity, but prevent twitching (Aukema *et al.*, 2005). The conformational change in the pilin subunits of the pilus (due to interaction with the PAIs) may relay a signal to PilT (possibly through the AIRNLIRE motif) to stabilize the conformational change (Aukema *et al.*, 2005). The conclusion is not that quorum-sensing modulates twitching motility but rather suggest that PAIs modulate pilus-functions independent of genetic control and that functional ATPases are required for pilus modulation *in vivo*.

Interestingly, when PAIs were added to PAK wild type cells attached to stainless steel, the result was a concentration dependent detachment (Figure 4-5-A). Therefore, if the *P. aeruginosa* cells are not attached to a surface, the PAIs modulate the pilus to increase the binding capacity with the intracellular ATPases acting as secondary messengers to stabilize the conformation change. Alternatively, if *P. aeruginosa* cells are bound to a surface the PAIs modulate the pilus to decrease the adherent capacity again through a conformational change induced by the PAIs and stabilized by the ATPases. The inability of PAK R364 *pilT* to respond to PAIs and detach from stainless steel suggests a reason for the observed inability of R364 to disperse from maturing biofilms (Figure 4-7-B) (Chiang and Burrows, 2003b). However, type IV pili are not necessarily required for dispersal from biofilms as dispersal is still induced in a pilus-deficient *P. aeruginosa* strain by nutrient conditions (Sauer *et al.*, 2004). Furthermore, nutrient mediated dispersal was associated with an increase in *fliC* transcription and a decrease in pilus expression, suggesting that flagella are important for dispersal (Sauer *et al.*, 2004). Therefore, the results of the present study may have more relevance during the formation and maturation of a biofilm than for dispersal in mature biofilms. There is no explanation for the increase in attachment of PAK S34 *pilU* mutants after the addition of PAIs post colonization (Figure 4-7-C). Although both PilT and PilU are required for twitching motility these proteins cannot substitute for each other and are functionally distinct (Burrows, 2005). Furthermore, PAK R364 *pilT* mutants are pilus-phage resistant, whereas, PAK S34 *pilU* mutants are pilus-phage sensitive, indicating that these proteins are not redundant (Whitchurch *et al.*, 1991; Whitchurch and Mattick, 1994). In addition, it has been reported that PilT localizes to both *P. aeruginosa* cell poles, whereas, PilU is only located at the piliated *P. aeruginosa* pole (Chiang *et al.*, 2005). The observed differential responsiveness of these two twitching motility deficient PAK strains is therefore not unprecedented.

Therefore, the PAIs at physiological concentrations may directly modulate the pilus structure, through a conformational change to increase or decrease the affinity of the pilus-tip for a variety of substrates. In addition to their ability to modulate pilus functions, pre-incubation of *P. aeruginosa* cells with PAIs accelerated microcolony formation (Figures 4-2-C and 4-2-E). Normally microcolonies develop at 5 to 7.5 hours after inoculation (O'Toole and Kolter, 1998) and are visually apparent after 24 hours (Singh *et al.*, 2000). In this report pre-incubation of huge densities of *P. aeruginosa* with PAIs causes the formation of distinct microcolonies after 1 hour (Figures 4-2-C and 4-2-E). Pre-incubation with PAIs causes a visual jump from single *P. aeruginosa* cell attachment to biofilm maturation stage-1, defined by a cell cluster thicker than 10 µm (Sauers *et al.*, 2002). Although PAIs increase the binding of pili to surfaces, PAIs do not cause pilus aggregation, suggesting that the ability to induce microcolony formation occurs by genetic regulation. However, no specific quorum sensing controlled genetic targets have currently been identified as relevant to the formation of biofilms (Reisner *et al.*, 2005). A recent study determined that DNA was released from *P. aeruginosa* cells through a quorum-sensing dependent mechanism and that microcolonies contain large amounts of extracellular DNA (Allesen-Holm *et al.*, 2006). This may explain the accelerated microcolony formation observed on addition of exogenous PAIs to high *P. aeruginosa* cell densities (Figure 4-2-C and 4-2-D). However, the results obtained by the addition of exogenous DNA did not result in the same acceleration of microcolony formation as addition of PAIs (Figure 4-8-C and 4-8-E). This suggests that although DNA causes aggregation of *P. aeruginosa* cells and is released in response to PAIs (Allesen-Holm *et al.*, 2006), there are still other factors involved in the accelerated microcolony formation observed due to the addition of exogenous PAIs.

Investigation into the effects of DNA on the initial colonization provide some explanation for the importance of DNA during initial biofilm formation (Whitchurch *et al.*, 2002). *P. aeruginosa* pili have recently been demonstrated to bind DNA from a variety of sources in a sequence independent manner (van Schaik *et al.*, 2005). Exogenous DNA increases the colonization of *P. aeruginosa* cells to stainless steel (Figure 4-8-C and 4-9-A) and inhibits the colonization to BECs (Figure 4-10-A) at certain concentrations. The ability to increase the colonization of *P. aeruginosa* to stainless steel by exogenous DNA is dependent on the type IV pili (Figures 4-8-C, 4-8-D, and 4-8-E). Pilus-mediated DNA binding is a multivalent interaction that potentially involved a groove formed by the β-sheet domain (van Schaik *et al.*, 2005). In contrast pilus-mediated binding to BECs and stainless steel is dependent on the C-terminal RBD (Giltner *et al.*, 2006; Irvin *et al.*, 1989). As the aspects of the pilus that are required for binding to stainless steel and DNA are distinct cellular aggregation is possible (Figure 4-8-E) and this provides an explanation for why DNA causes aggregation of *P. aeruginosa* cells (Allesen-Holm *et al.*, 2006).

I propose that the interaction between *P. aeruginosa* cells and DNA is similar to an antibody-antigen interaction. Therefore, an excess of *P. aeruginosa* cells and a small amount of DNA will produce no effect. Equivalent concentrations of *P. aeruginosa* and DNA will increase colonization due to aggregation of the *P. aeruginosa* by DNA. In contrast, an excessive concentration of DNA will inhibit the colonization *P. aeruginosa* cells. The results with DNase I also support this conclusion as the level of DNA present in the environment contributes to the colonization of *P. aeruginosa* cells to stainless steel (Figure 4-9-B) and BECs (Figure 4-10-C). This indicates that DNA may also be important for initial colonization of surfaces in addition to the requirement for DNA during biofilm development (Whitchurch *et al.*, 2002) and as a component of the EPS in mature biofilms (Matsukawa and Greenberg, 2004b). Consequently, the release of DNA through membrane blebs (Kadurugamuwa and Beveridge, 1995; Renelli *et al.*, 2004) or through the quorum-sensing dependent mechanism (Allesen-Holm *et al.*, 2006) may aid both the initial colonization of surfaces and completion of biofilm formation by *P. aeruginosa* (Whitchurch *et al.*, 2002).

Although an increase in *P. aeruginosa* cellular adherence mediated by the PAIs has implications for some types of acute infections, the ability of PAIs to increase pilus-mediated binding to DNA is probably more relevant to chronic CF lung infections. It has been established that *P. aeruginosa* forms biofilms in the CF lung that are not directly associated with the airway epithelium (Singh *et al.*, 2000). Furthermore, there is a very high concentration of DNA in the lungs of CF patients due to neutrophil necrosis (Lethem *et al.*, 1990; Sheils *et al.*, 1996). Recently, it was demonstrated that *P. aeruginosa* biofilm formation in the presence of neutrophils is enhanced due to actin and DNA polymers (Walker *et al.*, 2005), and several reports have demonstrated the importance of DNA in the initial stages of *P. aeruginosa* biofilm formation (Nemoto *et al.*, 2003; Whitchurch *et al.*, 2002). PAI concentrations associated with biofilms were observed to significantly enhance pilus-mediated binding to DNA (Figure 4-5) and Allesen-Holms (2006) recently demonstrated that DNA causes aggregation of *P. aeruginosa* cells. In addition, the results obtained with the addition of exogenous DNA during pilus-mediated binding to BECs (Figure 4-10-C) and asialo-GM$_1$ (Figure 4-10-B) indicate that the amount of DNA found in the CF lung would inhibit cellular binding. Therefore, it appears likely that the *in situ* PAI concentrations in the CF lung facilitate the saturation of DNA binding sites on the pilus, which may promote the formation of biofilms that are not directly associated with the airway epithelium.

Therefore, PAIs modulate the pilus directly by a proposed allosteric mechanism to increase binding and cause microcolony formation in a pilus-independent manner. In addition, DNA causes aggregation of *P. aeruginosa* cells (Allesen-Holm *et al.*, 2006) in a pilus-dependent manner, which either increases or inhibits colonization of *P. aeruginosa* cells depending in part on the affinity of the pilus for the surface. Therefore, the ability of PAIs to modulate binding to both surfaces and DNA, as well as the ability of DNA to aggregate *P. aeruginosa* cells, has major implications for biofilm formation and infection.

Chapter 5

General Discussion

5. General Discussion

5. 1. Pilus-mediated DNA binding and biofilm formation

This thesis has demonstrated that the type IV pili from several strains of *P. aeruginosa* could bind directly to DNA in a sequence independent manner (Figures 2-4, 2-5, and 2-7) (van Schaik *et al.*, 2005). The ability to bind DNA was a function of an intact pilus and was a multivalent interaction that can cause aggregation of *P. aeruginosa* cells (Figures 2-4, 2-10, and 4-8-E) (Allesen-Holm *et al.*, 2006; van Schaik *et al.*, 2005). Antibody inhibition assays using MAb PK99H and MAb PKL1 suggested that the C-terminal RBD is not directly involved in DNA binding (Figure 3-5). However, the ability of the PAIs to modulate the C-terminal RBD, potentially through a conformational change, increase the affinity of *P. aeruginosa* type IV pili for DNA (Figure 4-5). I propose that the pilus-DNA interaction is mediated by the 4-stranded anti-parallel β-sheet domain of the pilin that forms a groove along the length of the pilus and contains a conserved threonine residue (Thr98) (Figure 2-1). Therefore, the modulation of pilus-mediated DNA binding by the PAIs potentially occurs through stabilization of the connecting loop adjacent to the C-terminal RBD and the C-terminal RBD in a conformation away from the 4-stranded anti-parallel β-sheet domain of the pilin (Figure 3-8). The ability to move these loops away from the groove along the length of the pilus would remove potential steric interference and facilitate an increased affinity for DNA.

The ability of the type IV pilus to bind DNA has major implications for biofilm formation as *P. aeruginosa* biofilm formation is dependent on exogenous DNA and DNA is a major component of the biofilm EPS (Matsukawa and Greenberg, 2004a; Nemoto *et al.*, 2003; Whitchurch *et al.*, 2002). Evidence presented in this thesis has determined that DNA caused aggregation of *P. aeruginosa* cells in a pilus-dependent manner that either increased or decreased colonization of stainless steel and BECs (Figures 4-8-C, 4-8-E, 4-9, and 4-10). Therefore, the ability of *P. aeruginosa* to release exogenous DNA may facilitate colonization of surfaces and biofilm formation (Allesen-Holm *et al.*, 2006; Kadurugamuwa and Beveridge, 1995). As the interaction between DNA and *P. aeruginosa* is hypothesized to mirror an antibody-antigen interaction, the release of DNA at concentrations that cause aggregation of *P. aeruginosa* would facilitate colonization of a surface and also accelerate biofilm formation. In addition, the large concentrations of DNA in the lungs of CF patients may accelerate biofilm formation independent of cellular adherence (Brinkmann *et al.*, 2004; Lethem *et al.*, 1990; Sheils *et al.*, 1996; Singh *et al.*, 2000; Tomkiewicz *et al.*, 1993). Concentrations of DNA that are found in the lungs of CF patients were demonstrated to inhibit cellular adherence by *P. aeruginosa* (Figure 4-10). This data suggests an additional reason for the increased susceptibility of people suffering from CF to chronic *P. aeruginosa* infections. The amount of DNA found in the CF lung would aggregate *P. aeruginosa* and inhibit cellular adherence as the affinity for DNA is much higher than for epithelial cells (Irvin *et al.*, 1989; van Schaik *et al.*, 2005). A form of recombinant DNase I called Pulmozyme (Roche

Pharmaceuticals) is used to as a treatment by approximately 40% of CF patients. Treatment with Pulmozyme results in decreased sputum viscosity and improved lung function (Geddes and Shah, 1999) and may also prevent the formation of *P. aeruginosa* biofilms (Whitchurch *et al.*, 2002).

Therefore, the ability of the type IV pili from *P. aeruginosa* to mediate DNA binding can facilitate biofilm formation and colonization of a variety of surfaces (van Schaik *et al.*, 2005; Whitchurch *et al.*, 2002).

5. 2. Cis/trans isomerization of a conserved proline may mediate the allosteric regulation of the *P. aeruginsoa* type IV pili

Evidence was presented in this thesis suggesting there is a conformational change that can be induced in the *P. aeruginosa* type IV pilus, which modulates that affinity of the pilus for several substrates. The data presented on the characterization of MAb PKL1 suggests that binding of this monoclonal antibody altered the structure of the C-terminal RBD so that MAb PK99H could no longer recognize its epitope (Figure 3-3). In addition, the ability of the quorum sensing PAIs to alter the affinity of the type IV pilus for several different substrates also indicates that the PAIs could cause a conformational change in the C-terminal RBD (Figures 4-1, 4-3, and 4-5). One of the major functions of type IV pili is to facilitate twitching motiliy (Mattick, 2002). Interestingly, a recent study demonstrated that the C-terminal RBD specifically mediates binding to a variety of abiotic surfaces (Giltner *et al.*, 2006). Therefore, the structure of C-terminal RBD may have evolved to retain the ability to bind all the surfaces colonized by

P. aeruginosa. Alteration of the C-terminal RBD to mediate detachment from surfaces has major implications for twitching motility (Figure 4-7-A). Modulating the release of the pilus tip during twitching motility rather than breaking off the pilus and loss of pilin subunits, would be more efficient and would allow all pilin subunits to be recycled into new pili on the bacterial surface (Touhami *et al.*, 2006).

The hypothesized conformational change evolved from the observation that there is a conserved proline residue within the C-terminal disulfide bonded loop of various type IVa pilins from different species (Hahn, 1997; Hazes *et al.*, 2000). Studies using synthetic C-terminal RBD peptides provides insight into the proposed cis/trans isomerization around the imide bond between Ile138 and Pro139 of PAK (Campbell *et al.*, 1997; McInnes *et al.*, 1994; Wong *et al.*, 1995). The C-terminal RBD forms two β-turns, a type I β-turn (residues 134-137) followed by a type II β-turn (residues 139-142) (Campbell *et al.*, 1997). The PAK(128-144)ox peptide is found in both trans and cis forms in a ratio of 3:1 (McInnes *et al.*, 1994). Interestingly, substitution of Pro139 in the type II β-turn with an alanine decreases the peptide's affinity for epithelial cells, while also causing the disappearance of the cis form of the peptide (Wong *et al.*, 1995). This suggests that the cis form of the peptide is more important for cellular adherence. Several of the residues that are important for cellular adherence show large chemical shifts between the cis and trans peptide conformations including Gln136, Ile138, and Gly141 (Wong *et al.*, 1995). Even though all of the pilin structures solved to date (Audette *et al.*, 2004a; Craig *et al.*, 2003; Hazes *et* *al.*, 2000; Parge *et al.*, 1995) are in the trans conformation, this could be due to the crystallization conditions, while the pilin structures may adopt a cis conformation under different crystallization conditions (Andreotti, 2003). Particularly as the above data using synthetic peptides suggest that the cis conformation is more important for cellular binding (Campbell *et al.*, 1997; Wong *et al.*, 1995).

Cis/trans proline isomerization occurs in several folded proteins and has important implications for signaling control. A proline switch, present in the SH2 domain of interleukin-2 tyrosine kinase, modulates ligand recognition (Mallis *et al.*, 2002). The location of this proline within a loop minimizes steric constraints and is probably a favorable feature for a proline switch (Mallis *et al.*, 2002), which is similar to the position of Pro139 within the C-terminal RBD. The most similar structures to type IVa pilins are the major coat proteins of icosahedral RNA phages including MS2 (van Schaik *et al.*, 2005). A lateral gene transfer event may have occurred to produce the pilin structural subunit. Cis/trans proline isomerization occurs within the coat protein of the MS2 bacteriophage (Golmohammadi *et al.*, 1993). However, the cis/trans isomerization in MS2 does not occur in the same loop as in the *P. aeruginosa* pilin. The MS2 cis/trans isomerization would occur in the loop adjacent to the C-terminal RBD in the pilin structure (Golmohammadi *et al.*, 1993; Hazes *et al.*, 2000). However, the proline isomerization in the MS2 coat protein causes changes in the adjacent loop, which would correspond to the C-terminal RBD of *P. aeruginosa* pilin (Golmohammadi *et al.*, 1993; Hazes *et al.*, 2000). Therefore, the PAIs and MAb

PKL1 potentially bind and alter the structure of this adjacent loop, which causes the cis/trans Pro139 isomerization in the C-terminal RBD to increase or decrease binding. This increase in binding may result from the disappearance of the type I β-turn from the C-terminal RBD in the cis conformation, and other structural changes involving important residues for binding, such as Gln136, Ile138, and Gly141 (Campbell *et al.*, 1997; McInnes *et al.*, 1994; Wong *et al.*, 1995). Interestingly, NMR data colleted with K122-4 pilin was unable to determine the structure of the second β-turn or residues 139-142 as there was spectral overlap between Pro 139, Lys 140, and Thr141 (Keizer *et al.*, 2001), supporting the possibility of a conformational change in the native pilin. In addition, a NMR backbone dynamic study demonstrated that there may be isomerization of the disulfide bond between C129 and C142 (Suh *et al.*, 2001), which could result from cis/trans isomerization of Ile138-Pro139 imide bond. The trans to cis interconversion requires 0.61 kcal/mol in the oxidized PAK peptide (McInnes *et al.*, 1994), which is less than the 14-24 kcal/mol required for conversion in an unfolded protein (Andreotti, 2003). Therefore, it is reasonable that binding of the PAIs or MAb PKL1 to the loop adjacent to the C-terminal RBD may create new hydrophobic or hydrogen bond interactions that could provide the interconversion activation energy necessary for a proline switch. Consequently, the PAIs and MAb PKL1 would act similar to a cis/trans isomerase to stabilize the transition state between the cis and trans conformers (Andreotti, 2003).

Therefore, I propose that a cis/trans isomerization of the Ile138-Pro139 imide bond modulates that affinity of the *P. aeruginosa* type IV pilus. This conformational change is potentially important for twitching motility and maturation of biofilms.

5. 3. Future directions

As pilus-mediated DNA binding is important for biofilm formation on abiotic surfaces and potentially for biofilm formation in the CF lungs, it will be important to determine what structural aspects of the pilus mediate this interaction. I imagine that the conserved Thr98 present in the β-sheet structure of many type IV pili is required for DNA binding. Interestingly, in the closely related MS2 coat protein structure dimers bind RNA through direct interaction of the β-sheet residues, and where Ser and Thr side chains contribute directly to RNA binding (Helgstrand *et al.*, 2002). Creation of a point mutation Thr98 to Ala in the cloned full length K122-4 pilin structure would allow expression in a non-piliated strain of *P. aeruginosa* to determine if DNA still increases colonization or causes aggregation of the cells. This would determine if the conserved threonine is required for DNA binding and suggest that DNA binding is a conserved function of type IV pili in many bacterial species.

The ability of *P. aeruginosa* type IV pili to bind directly to DNA has major implications for chronic CF lung infections. Evidence presented in this thesis suggests that the high concentration of DNA in the lungs of CF patients may cause accelerated biofilm formation in a type IV pilus-dependent manner (Figure 4-10). Animal model studies could be performed to determine if the colonization of *P. aeruginosa* in the presence of high concentrations of DNA leads to a chronic rather than acute infection. Evidence presented in this thesis suggests that the high concentration of DNA in the lungs of CF patients may cause accelerated biofilm formation in a type IV pilus-dependent manner (Figure 4-10). The rat model for *P. aeruginosa* pneumonia could be used to determine if DNA can cause the switch to chronic colonization (Vanderzwan *et al.*, 1998).

It was demonstrated that pilus-mediated binding functions are altered by PAIs and have suggested that this occur by a proline switch in the C-terminal RBD (Figures 4-1, 4-3, and 4-5). The identification of cis/trans proline isomerization in folded protein is difficult as most biochemical methodologies are unable to detect this change (Andreotti, 2003). However, as NMR is capable of detecting a proline switch, NMR studies will be carried out with PAK pilin and PAK pilin after binding by MAb PKL1 antibodies. Alternatively, NMR studies will be performed with PAK pilin and PAK pilin in the presence of PAIs. In addition, to determine if the cis conformation of the C-terminal RBD mediated the binding functions of the pilus site-directed mutagenesis of Pro139, which causes the disappearance of the cis conformer in the PAK(128-144)ox peptide (Wong *et al.*, 1995) could be performed in the cloned full length PAK pilin and then expressed in a non-piliated strain of *P. aeruginosa*. A difference in the ability to bind to BECs compared to a non-piliated strain expressing the wild type full length PAK pilin will determine if the cis form of the C-terminal RBD mediate the binding functions of the type IV pilus.

These studies will help to further elucidate the structure-function relationship of the *P. aeruginosa* type IV pili and may provide general information on the function of type IV pili.

Chapter 6

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